PHYSIOLOGICAL MARROW OSSIFICATION IN FEMALE PIGEONS

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Structural changes occur in the bone marrow of female pigeons which are not found in the male. Such changes are, essentially, variations in ossification of the marrow and are coincident with changes in the ovary.

Our observations were made upon the red marrow of the long bones of the leg and extending over a period of 2 years relate to 850 pigeons—449 male and 401 female. Observations were made in all months of the 2-year period.

By gently splintering the shaft of the femur freshly removed form a male pigeon, the marrow may be exposed in situ as a dark red, moist, pulpy cord completely filling the medullary cavity. No appreciable attachment of this cord to the inner surface of the shaft exists and the marrow may be removed in toto, exposing the inner surface of the bone as smooth and glistening. This condition is constant for all male pigeons.

In sharp contrast to the uniformity of the marrow pattern thus presented in the male is the extreme individual variation seen in the female. For although a considerable number of females killed at random present a marrow pattern indistinguishable from that of the male, an equal number show an ossification of the marrow. Histologically, this is a true ossification displaying lamellation and haversian canals. The degree of ossification in the female differs markedly in individual instances. In certain females it is slight and is limited

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to delicate spicules of bone extending from the inner surface of the shaft a short distance into the marrow. In such instances the removal of the marrow exposes the wall of the medulla as a rough granular surface in contrast to the smooth glistening surface seen in the male.

In certain other females delicate trubeculae of bone extend well into the marrow and the marrow cannot be stripped from the medullary canal. With this medium degree of ossification, the marrow remains moist and of dark red color.

In those females where the ossification is maximal, anastomosing trabeculae of bone extend in every direction through-out the medulla, the marrow proper being enmeshed in the interstices of the bony network. The marrow is relatively dry, its color is gray and the texture is chalky.

While for convenience in description the above degrees of slight, medium, and extreme ossification are cited, all intermediate degrees of ossification occur with the shading of the one group into the next.

Of the 449 males observed, none showed a marrow ossified in any degree. Of the 401 females observed, 215 showed marrow without ossification; 112 showed slight or medium marrow ossification and 74 showed extreme ossification.

The fact that the bone marrow of female pigeon undergoes osseous modifications which do not occur in the male is of less import than the fact that these changes are cyclic and parallel functional changes of the ovary. The degree of ossification varies directly with the size of the developing ovarian follicles. Thus when the ovary contains no follicle of a diameter greater than 2 mm. there is no ossification of the marrow. But when the ovary contains a follicle of more than 4.5 mm. in diameter, there is always some degree of ossification of the marrow and when a 10-mm. follicle is present there is always extreme ossification of the marrow.

Systematic measurement was made of the diameter of the largest ovarian follicle found in each of 298 females and these diameters averaged for each of the three groups showing: no ossification, medium ossification and extreme ossi-

fication: the mean diameter of the maturest follicle in those females with no marrow ossification was 1.6 mm.; for those with medium ossification, 4.1 mm. and for those displaying extreme ossification, 9.1 mm.

We conclude from these findings that in female pigeons there is a cyclic ossification of the red marrow of the long bones of the leg and that the ossification is coincident with the maturation of the ovarian follicle.

THE ADRENOTROPIC SUBSTANCE OF THE HYPOPHYSIS AS INFLUENCED BY AGE, CASTRATION, SEX AND THYRO-PARATHYROIDECTOMY [1]

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TWO FIGURES

INTRODUCTION

A decade ago little was known about the function of the anterior hypophysis. In 1924, Evans described the growth hormone and showed that giant rats could be produced with this extract. A clear demonstration of the sex hormone was given by Smith and Engle ('27) and Zondek and Aschheim ('27). Several other reactions of the anterior hypophysis soon were published; so that by 1931, Bugbee et al. were able to list eleven distinct physiological functions of the anterior hypophysis. Since then several other functions have been described. During the past year, studies on the cortico-adrenal stimulating substance of the hypophysis have been made in this laboratory (Emery, '33 a; Emery and Atwell, '33 a, b). The hypertrophy of the adrenals was readily obtained with an extract of the whole sheep pituitary gland.[2] Similar results have been reported by Robson and Taylor ('32) and Fluhmann ('33). Our previous experiments with pituitary implants (Emery, '33 a, b) had given evidence of adrenal hypertrophy, but to a lesser degree than that obtained with extracts. The fact that the donors of these implants were all castrated rats, seemed to be a point for further

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[2] Kindly supplied by Doctor Kamm, Parke, Davis & Co.

investigation. This will be discussed in the present paper, which deals with castration, sex and thyroparathyroidectomy in relation to hypertrophy of the adrenals.

MATERIAL AND METHODS

The adrenal hypertrophy was studied in albino rats. Approximately 100 animals were used as recipients of implants, fifty were injected with pituitary extracts, and a similar number kept as normal controls. More than 700 rats and guinea pigs were donors of pituitary implants.[3] The beef pituitary glands were obtained from a local abattoir. The whole sheep pituitary gland extract was supplied by Parke, Davis & Co. Implants were usually made in the muscles; in a few cases, they were placed intraperitoneally. The anesthetic was ether. In the case of the beef pituitaries, these were minced when fresh and kept frozen until ready to use, but never kept longer than a few weeks.

The experiments differed somewhat in each series of rats, but, in general, the implants or extracts were either given daily or at least three treatments during a period of 10 days. On the day following the last treatment the animal was killed and the several organs, dissected free from other tissue, were weighed.

RESULTS

Hypertrophy at different ages

The small amount of adrenal hypertrophy produced by pituitary implants in rats less than 40 days of age (Emery, '33 a) was quite in contrast to the results obtained with extracts in larger rats (Emery and Atwell, 33 a, b). In the former the increase in adrenal weight was not more than 25 per cent, while in the latter it was in many cases more than 100 per cent. This led to the present consideration of age as a factor in adrenal hypertrophy. Rats at different ages

[3] The authors were able to use discarded animals from several sources, but especially are we indebted to Dr. S. L. Vaughan, of the Buffalo General Hospital, Dr. E. B. Hanan, of the Buffalo City Hospital, and Dr. S. W. Kletzien of the Gratwick Laboratory.

were given whole pituitary gland extract in daily doses of ½ cc. per 100 gm. body weight. The results are shown in table 1. The number of animals at each age was not less than four. The variation in the amount of hypertrophy, although considerable, is not confined to any age group. Animals from 3 months to 2 years of age responded to the injections in a similar manner. In young rats at weaning age the hypertrophy is more difficult to obtain and we have failed to obtain it when the injections were started at 20 days of age.

TABLE 1

Per cent of hypertrophy of the adrenal glands at different ages in rats produced by ten injections of whole pituitary extract of sheep (Parke, Davis & Co.). Dose ½ cc. per 100 gm. body weight per day. Four to ten rats in each group

AGE IN MONTHS	PER CENT HYPERTROPHY	
	Normal males	Normal females
1	0	0
3	50	29
6	45	33
9	30	—
12	38	27
15	25	47
24	—	29

In sexually mature animals the adrenal weights were more variable in females than in males. This variation was reduced by the selection of virgins and females that had not suckled young for some weeks. Lactation, although known to increase the weight of the adrenal glands (Andersen and Kennedy, '32), was, therefore, not a factor in these experiments.

Effect of castration

That castration increases the sex hormone of the hypophysis of rats, as shown by Engle ('29) and later by many investigators, is now accepted.

In the previous reports (Emery, '33 a, b) of hypertrophy of the adrenal glands produced by pituitary implants the

donors were castrated rats. This fact has been largely responsible for a further study of the problem. Subsequent experiments, however, show that castration played only a minor role in these earlier experiments. The number of castrated rat pituitary glands necessary to cause adrenal hypertrophy was found to be eight or more. This has prevented the use of a large number of recipients. The results, however, as shown in table 2, have failed to establish that the hypertrophy was greater in amount when castrated rats were used as donors. It, therefore, seems certain that castration has no apparent effect on the adrenotropic substance of the hypophysis. The trauma and irritation incidental to the implants could easily account for a slight hypertrophy and tend to make the adrenal weights more variable. This non-specific effect on the weight of the adrenals, we have previously encountered with brain tissue extracts (Emery and Atwell, '33 b) and it is likewise evident in the guinea pig brain tissue implants shown in table 2.

The experiments with beef pituitary glands have likewise shown that castration apparently does not increase the adrenal-stimulating potency of the pituitary (table 3). A clearer demonstration of the effect of castration in bovines would be to compare the pituitary glands of gonadectomized cattle to the normals of the same sex. This we have not had the opportunity to do in cattle, and our data on this point are confined to the rat experiments already discussed.

Effect of difference in sex of donors

It was interesting as well as surprising to find the female pituitary glands very potent as compared with the male. The virgin female rats used as donors weighed approximately 150 gm.; thus they had almost completed their growth. The fully matured multiparous females had even more potent pituitary glands than the virgins, as shown by 29.3 and 47.7 per cent increase in the adrenal glands of the recipients of these two groups (table 2). Also, the hypertrophy obtained with the pituitary glands of cows and heifers is much greater

TABLE 2

Body weights in grams. Other weights in milligrams. Recipients usually killed 10 days after first implant

DONORS FOR EACH RECIPIENT	RAT RECIPIENTS—AUTOPSY DATA					
	Number and sex	Body	Ovary	Thyroid	Adrenal	Per cent increase in adrenal weight
Rat						
8 old males	5 females	76	120.0	7.4	21.3 ± 0.603	22.2
8 old females	5 females	72	52.0	6.0	20.5 ± 0.202	17.1
8 castrated males	15 females	70	175.0	8.0	20.4 ± 0.431	16.6
None	15 controls	69	16.7	8.1	17.5 ± 0.495	
10 castrated males	5 castrated males	181		15.5	40.9 ± 0.944	25.1
10 old males	10 castrated males	182		12.4	37.4 ± 0.876	14.3
10 old females	7 castrated males	191		12.6	48.3 ± 0.568	47.7
10 old females castrated	5 castrated males	190		11.0	46.0 ± 2.020	40.7
10 virgin females	6 castrated males	195		10.0	42.3 ± 3.350	29.3
10 virgin females castrated	5 castrated males	189		12.1	38.4 ± 1.680	17.4
None	15 controls	197		16.8	32.7 ± 1.100	
Guinea pig						
8 females	2 old females	176	38.0	16.5	60.2 ± 4.690	43.3
None	10 old females controls	163	45.0	14.6	42.0 ± 1.340	
8 females	5 castrated males	182		11.8	44.9 ± 0.675	42.5
8 males	8 castrated males	174		10.7	39.5 ± 1.730	25.4
Pieces of brain tissue	5 castrated males	186		10.6	35.5 ± 4.690	12.4
None	25 controls	179		13.9	31.5 ± 0.900	

TABLE 3

Normal male rats transplanted with beef pituitaries

TRANSPLANT	NUMBER OF RECIPIENTS	AVERAGE WEIGHT IN GRAMS		
		Body	Testes	Adrenals
Cow, anterior lobe	4	178	1.96	0.0529
Heifer, anterior lobe	5	199	2.24	0.0515
Steer, anterior lobe	5	191	1.94	0.0360
Beef, posterior lobe	6	156		0.0339

than that obtained in similar experiments with the pituitary glands of steers. The mean adrenal weights were 52.9, 51.5 and 36.0 mg. for the three groups, respectively (table 3). The weight of the adrenals of the recipients, 36 mg., in the steer group was similar to the weight of 33.9 mg. found in the control group implanted with posterior lobe tissue.

Therefore, in cattle as in rats, the pituitary of the female is more potent for the adrenotropic substance. Likewise, this seems to be true in guinea pigs, as shown in table 2. The adrenal weights of the recipients receiving eight female donors averaged 44.9 mg. as compared to 39.5 mg. when male donors were used. The guinea pigs were supplied largely from the local hospitals and were usually dead, but in good condition when received. The females were from breeding cages, and therefore considered to be multipara. From these data, it is evident, as shown in table 2, that the female hypophysis possesses something which has considerable effect on the adrenal glands of recipient rats. These results are summarized in figure 1.

The influence of the thyroid gland

During the progress of these experiments we had on a few occasions injected thyroparathyroidectomized rats with a saline suspension of anterior lobes of pituitary glands containing the adrenal stimulating substance, and in every case the usual hypertrophy of the adrenal glands did not occur. We soon became aware of the experiments (Loeser, '33) in guinea pigs, in which removal of the thyroid gland prevented the hypertrophy of the adrenals. This led us to repeat our first observations. The results are shown in figure 2. Whenever the thyroid was completely removed, the usual hypertrophy of the adrenal brought about by pituitary extract did not occur. In one or two cases in which it did occur, it was found upon autopsy that the thyroidectomy had been subtotal; these cases are not included in the figure. Such variation as is shown in adrenal size in thyroparathyroidectomized rats is within the range of normal individual differences; it

appears, therefore, that the hypertrophy of the adrenal glands with anterior pituitary substance is conditioned by the presence of thyroid tissue in the organism.

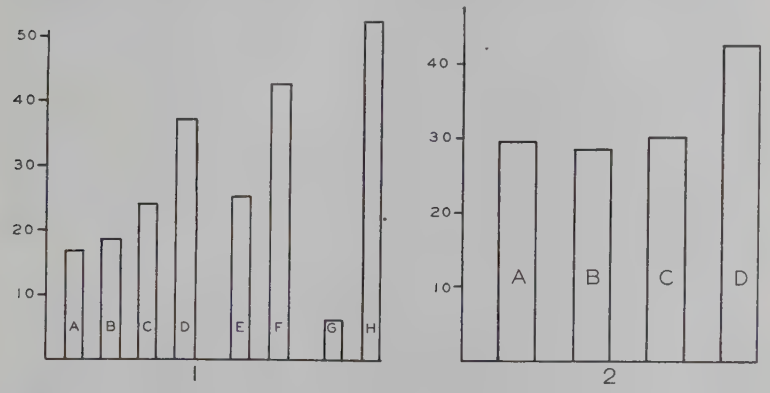

Fig. 1 Adrenal hypertrophy as influenced by sex of the donor. The height of each column represents the per cent of adrenal hypertrophy observed in rats receiving pituitary implants. Donors are as follows: A, normal male rats; B, castrated male rats; C, virgin female rats; D, multiparous female rats; E, male guinea pigs; F, female guinea pigs; G, steers; H, cows and heifers.

Fig. 2 Adrenal hypertrophy as influenced by thyroparathyroidectomy of the recipients. The height of each column represents the average weight in milligrams of the adrenals. A, normal male rats, untreated. B, thyroparathyroidectomized male rats, untreated. C, thyroparathyroidectomized male rats, injected with saline suspension of anterior lobe. D, normal male rats, injected with saline suspension of anterior lobe.

DISCUSSION

The hypertrophy of the adrenal glands seems to have little or no relation to the sex glands of the recipients. It has been the custom in previous work (Emery and Atwell, '33 b) to study the hypertrophy in castrated animals, and this we have continued in order that hypertrophy would not be incidental or secondary to the stimulation of the sex glands. Theelin has practically no effect on the adrenals (Leonard et al., '31); however, the recent report of Leiby ('33), in which considerable adrenal hypertrophy was produced with theelol leads one to believe that further experiments may show the gonads to have an effect on the adrenals. Yet the

effect of castration, per se, has very little effect on the adrenals, as the review by Andersen and Kennedy ('33) has shown.

In young rats, a certain age apparently has to be attained before the animals will respond to certain hormones of the pituitary. Corey ('28) reported that the sex hormone would not stimulate the ovaries of rats until after 10 days of age, and recently Hertz and Hisaw ('34) have shown that the ovaries of 4-week-old rabbits were unmodified after treatment with pituitary extracts. The activity of the pituitary adrenal-stimulating substance as judged by hypertrophy likewise has no effect until the rats are about 25 to 30 days of age.

It was surprising to find the female pituitary gland more potent than that of the male. The experience with the pituitary sex hormone from many laboratories has been that the male pituitary is several times more potent than the female. The sex hormone does not noticeably enlarge the adrenals, as shown by experiments using pituitary extracts potent for the ovarian-stimulating hormone, but lacking the adrenotropic substance. Likewise, Antuitrin S (from pregnant urine) has no effect on the adrenals as we have used it (Emery and Atwell, '33 b). Leiby ('33) has, however, reported adrenal hypertrophy after treatment with Antuitrin S. The view herein expressed that the adrenal hypertrophy is due not to the sex hormone, but rather to some other substance in the extract used, supports the view of Collip et al. ('33) that the adrenotropic and thyrotropic principles can be separated from the other substances in anterior lobe extract.

On the other hand, it is difficult to understand why saline suspension of fresh anterior lobe tissue does not produce adrenal hypertrophy in thyroparathyroidectomized rats if, as Collip et al. ('33) believe, the adrenotropic factor is separate from the thyrotropic principle. Although these authors present some evidence for the separation of the two principles, it would be interesting to observe the effect of the separated substances on thyroidectomized rats. It is not always easy to detect the presence of the thyrotropic principle by observa-

tions on the rat thyroid. The adrenal glands may double in weight after pituitary extract treatment and yet the thyroids show no hypertrophy (Emery and Atwell, '33 b). The metabolic rate may be increased by pituitary extract stimulating the thyroid and yet the histology of the thyroid remains normal (Fluhmann, '34). That the thyroid substance may stimulate the adrenal is shown by experiments in which thyroxine produced adrenal hypertrophy in rats (Gohar, '33) and in guinea pigs (Loeser, '33). Likewise, rats fed on beef thyroid had large adrenal glands (Herring, '17). Thus, it seems that the thyroid normally plays a part in adrenal hypertrophy and may even be the primary cause of it.

SUMMARY

1. The hypertrophy of the adrenal glands produced by pituitary implants or extracts has been studied in respect to age, sex, castration and thyroparathyroidectomy.

2. Approximately eight pituitary glands of rats or guinea pigs are required as implants to bring about hypertrophy of the adrenal gland in rats.

3. Adrenal hypertrophy was not obtained in rats under 30 days of age. In older rats, 3 to 24 months of age, age was not a factor in determining the amount of hypertrophy.

4. The pituitary glands of female rats, guinea pigs and cattle were more potent for the adrenotropic substance than the pituitaries of males. Castration of the donors had little effect.

5. The hypertrophy of the adrenal glands has so far not been obtained in thyroparathyroidectomized rats.

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POLYOVULAR FOLLICLES IN THE CAT

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THREE PLATES (ELEVEN FIGURES)

An additional instance is presented of the occurrence of polyovular follicles in the cat, previously noted by Schrön, Kennedy, Hartman, and others. Some authors have described them in the primordial stage, or in the later stages, with or without an antrum. In the material to be described, numerous cases of all three conditions were observed, and certain details of the ovum, the zona pellucida and the follicle cells could be clearly seen.

The material consisted of ovaries from a cat nearly 1 year old. One ovary, fixed in Bouin's fluid, which furnished a nearly complete series of 316 sections was found to contain twenty-one biovular, nine triovular follicles, and one follicle with five ova. From the other ovary, fixed in chrom-osmic, seventy-two sections were obtained. This series, although incomplete, yielded six biovular, six triovular follicles, and two follicles with five ova.

DESCRIPTION

The first figure (fig. 1), representative of the structure of the ovary, shows the cortical layer of primary follicles, the zone of graafian follicles, and the central vascular area. A biovular follicle is conspicuous at the upper right of the figure.

Primordial follicles

Figure 2 shows a portion of the cortex, containing masses of primordial ova. Hartman ('26) has described these cells

in the cat as forming a dense zone five or six rows deep lying close to the periphery, with many cells in close contact. They are only potentially polyovular, he believes, but their massing is significant. Among the primordial follicles in figure 2 may be seen a two-celled and three-celled grouping of ova, which strongly suggests the beginning of compound follicles. The cells are flattened against one another, the two ova lying apparently in direct contact, the three ova (figs. 2 and 3) being separated from each other by a few squamous cells. The flattening of the ova is not in itself significant, for this condition is quite common among the masses of cortical cells, but it is significant that in these two cases, which are typical of a number of others, the groups are separated from other ova by a considerable amount of connective tissue, though as yet no stromal capsule is developed.

In later ova (fig. 4) a narrow zona pellucida is clearly visible, and this increases in thickness with the growth of the ovum. In this biovular follicle the ova are separated along their flattened surface by a layer of squamous cells, which merge with the cuboidal cells of the follicle.

Rabl (1899) has figured a similar stage, in which, however, the two ova are in immediate contact. O'Donoghue ('12), working on Dasyurus, has stated that if a layer of cells lies between the ova, these are ordinary ova, and not destined for the same capsule. In the opinion of the writer, the presence or absence of intervening cells is unimportant, the main criterion being, as shown in this figure, the enclosure of two or more ova within a continuous follicle layer, and their isolation from other ova by a fairly large area of connective tissue.

Another variant of a compound follicle, which I interpret as a potential triovular follicle, is shown in figure 5. Here the ova, not compressed by contact, are surrounded externally by a continuous follicle layer, which merges with the interior cells separating the ova. The cells are in transition from cuboidal to columnar form, and the rudiment of a stromal capsule is visible. Beyond this lie masses of interstitial cells.

Growing follicles

Various stages in the growth of compound follicles are shown in figures 6 to 11. In figure 6, two ova of unequal size are surrounded by two or more rows of follicle cells. Figure 7, which appears essentially similar, is actually a triovular follicle, the third ovum appearing in adjacent sections. The size of the two ova shown is maximum in this section. The zona pellucida around the larger ovum is broad and conspicuous, its outer surface being reticular in appearance, where it is in contact with the follicle cells. These cells form a solid mass terminating in a layer of columnar cells bounded by the connective tissue capsule. It will be noted that the connective tissue merges with a layer of interstitial cells lying close against the upper part of the follicle. Kingsbury ('14), describing similar masses of interstitial cells encircling follicles, has shown that they originate from the connective tissue cells and later invade follicles which become atretic.

Three follicles were observed containing five ova of unequal size, lying in a compact mass of follicle cells (fig. 8).

In figure 9, a biovular follicle, two small follicular cavities are present. In figure 10 a considerable antrum separates the inner and outer layers of follicle cells, except in the region of the cumulus oophorus. The second ovum, which appears in the next section to this, lies in the center of the cell mass seen just above the ovum figured. As in figure 7, the follicle is partly encircled by interstitial cells.

The two largest follicles observed were triovular, and they happened to lie adjacent to each other (fig. 11). In the follicle on the right, the plane of section has cut through the nuclei and nucleoli of two ova. The position of the third ovum, which is in the adjoining section, is indicated by a clear central region in the lower mass of cells. In the follicle on the left, the third ovum is not visible, but it appears in another section, at the upper region of the follicle.

Each ovum in these two follicles has a conspicuous zona pellucida somewhat fibrillated on its outer surface, and each

lies embedded in its cumulus oophorus, separated from the follicular fluid by about three or four rows of cells. The sheathing cells of two ova are confluent at one point, but no cases were observed in which two ova had a cumulus in common, such as Hartman described in the cat. The follicle on the right corresponds very closely with the triovular follicle figured by Schrön (1863) from a cat 1 year old. He found only one biovular and one triovular follicle in a large number of ovaries examined.

FATE OF POLYOVULAR FOLLICLES

The graafin follicles described here cannot be considered as mature. The ova have no uniform orientation with reference to the surface of the ovary, and the follicles are located too deep within it to produce any bulging on its surface. According to Kingsbury ('13), Hartman ('26) and others, compound follicles are of common occurence in mammalian ovaries before the period of sexual maturity. These follicles disappear, and those of the adult period then develop. It is therefore probable that the follicles described here would not have furnished definitive ova, but would eventually have degenerated.

DISCUSSION

Kingsbury ('13) has described a peculiar development of follicle cells in both uniovular and biovular follicles in the cat. He makes a distinction between the sheathing cells immediately surrounding the ovum (traceable back to the squamous cells of the primordial stage) and the outer or investing follicle cells that form the boundary of the antrum.

These outer cells are derived, he believes, not by multiplication of the antecedent follicle cells, as is generally held, but from a nest of epithelial cells, which during their growth and multiplication become wrapped around the primordial follicle cells, finally enclosing them and becoming indistinguishable from them. In this process some stromal cells may be carried into the interior of the follicle. A follicular cavity appears

later. Three points of evidence offered in support of this interpretation are first, that, in general, the smaller eggs are less enclosed, the nest of cells surrounding only half or less of an ovum; secondly, the presence in the larger follicles of an apparent line of junction where the encircling cell mass had met and fused; thirdly, the inclusion of nodules of stromal cells within the follicular mass.

In the material described in this paper, all of the growing follicles were examined in detail, and no cases were observed of nests of such cells lying close to follicles, or in process of wrapping around them. Nor was there evidence of the inclusion of stromal cells within the follicle. In one doubtful case, a follicle containing five ova (fig. 8), a small mass, not of stroma, but of interstitial cells, appeared in the center of the group. Owing to the loss of a few sections at this point, the path of its inclusion could not be traced.

Another anomaly noted by Kingsbury was the extreme irregularity in the form of the later follicles. In my material, the contours of the older follicles varied from the common rounded or elliptical forms to those flattened on one side or even somewhat incurved (figs. 1 and 11), but no cases of extreme irregularity were observed.

CONCLUSION

The evidence from the foregoing study leads to the conclusion advanced by Schrön, Hartman and others that polyovular follicles arise through the failure of the connective tissue to grow between the ova and isolate them. It appears that in early compound follicles the ova, surrounded by a single layer of cells, may be either in direct contact as figured by Rabl, or separated by a layer of follicle cells, as in figures 3 to 5. Given the smoothly continuous follicle layer around two or more ova, and a certain isolation from other ova, it would seem that an incipent compound follicle results. The later development of a compound follicle proceeds as in a normal one, the cells, after becoming columnar, multiplying and producing a solid mass, surrounded now by a definite capsule

of rearranged connective tissue elements (figs. 6 and 7). One or more small cavities appear (fig. 9) which increase in extent, eventually forming the antrum (figs. 10 and 11). The relation of ovum to cumulus and to granulosa appears entirely normal. Except for the large number of ova, there were no anomalies observed in the development of the follicles.

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PLATES

EXPLANATION OF PLATES

Photomicrographs by Jean Kieffer

Magnifications are: figure 1, × 40; figure 11, × 75; figures 2, 6, 7, 8, 9, 10, × 150; figures 3, 4, 5, × 300.

PLATE 1

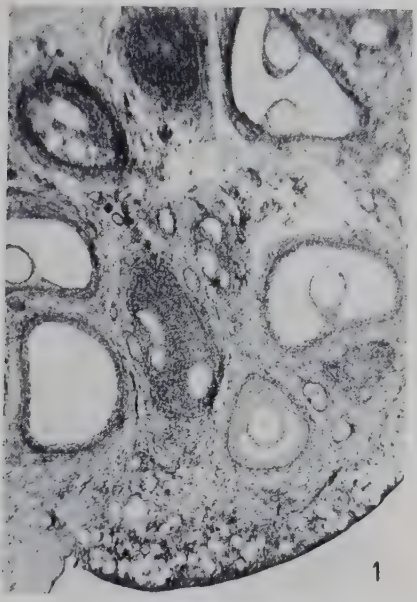
EXPLANATION OF FIGURES

1 Portion of cross section through ovary, showing cortical layer of primordial follicles, a zone of graafian follicles, and a central vascular area. A biovular follicle at upper right of figure.

2 Portion of cortex, with primordial follicles. a, b, groups of 2 and 3 cells surrounded by a layer of squamous cells. The connective tissue is somewhat torn near b.

3 Same group of three cells, enlarged.

4 Two ova in single follicle of cuboidal cells, no other ova near. z, zona pellucida. Connective tissue slightly torn at left.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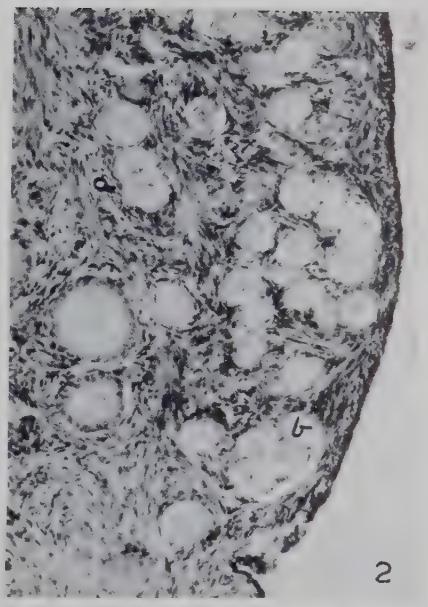
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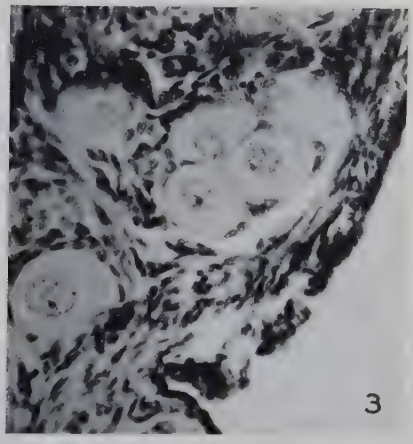
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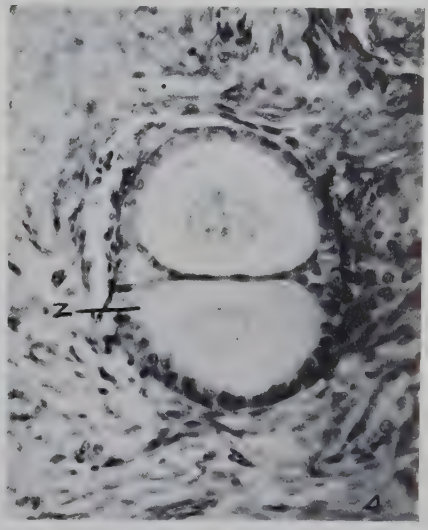
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PLATE 2

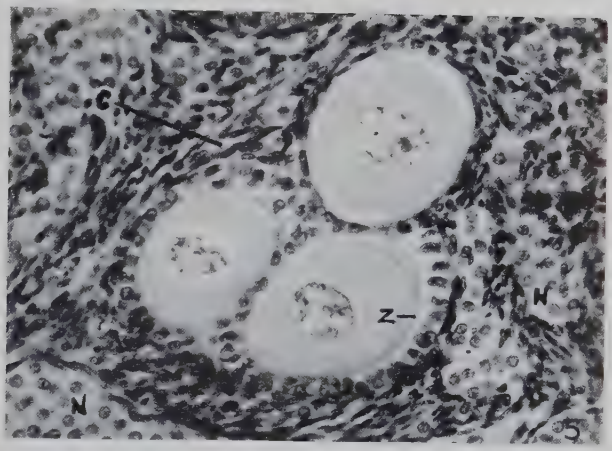
EXPLANATION OF FIGURES

5 Group of three ova, surrounded by follicle cells in transition from cuboidal to columnar, and from one layer to two layers of cells. Zona pellucida (z) conspicuous; c, stromal capsule developing; n, masses of interstitial cells. No other ova near.

6 Two ova of unequal size, surrounded by two or more rows of follicle cells.

7 Triovular follicle. Two ova of unequal size; the third ovum, the size of the smaller one, lies in the next section. z, zona pellucida; c, stromal capsule; n, interstitial cells. Cortical zone at upper right.

8 Follicle with five ova of unequal size.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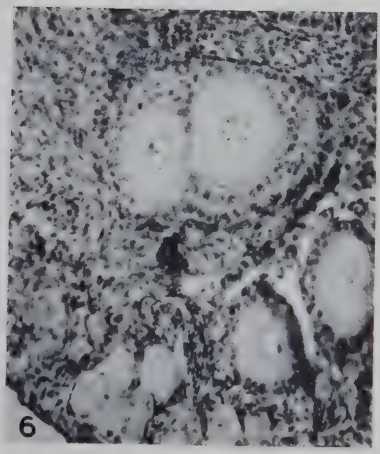
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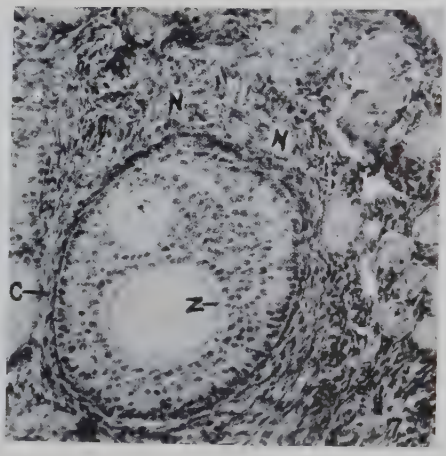
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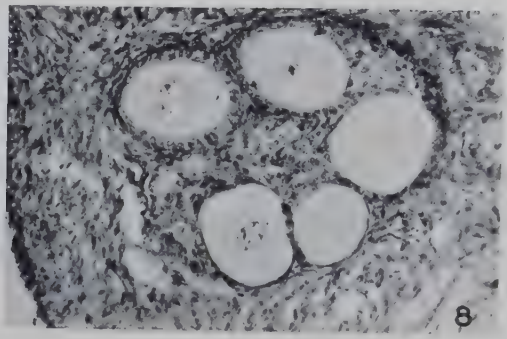
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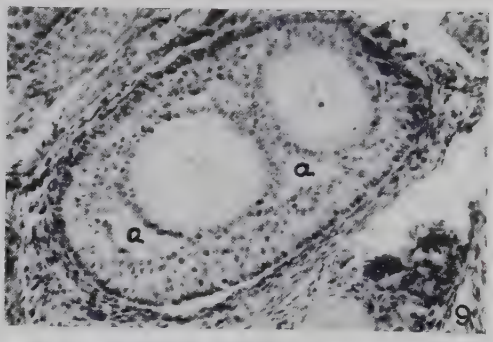
PLATE 3

EXPLANATION OF FIGURES

9 Biovular follicle, showing beginning of follicular cavity. Stromal capsule torn at upper right.

10 Biovular follicle, with antrum separating inner and outer layers of follicle cells. x, position of the second ovum, which lies in the next section.

11 Two triovular follicles, with large antrum. In each case only two ova are visible. x, position of third ovum, which lies in the next section; s, surface of the ovary.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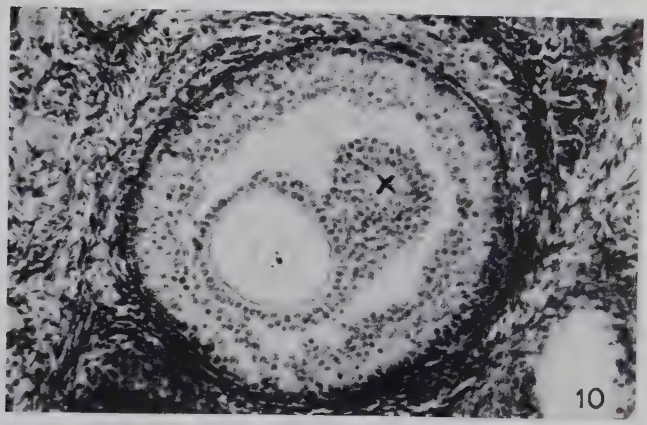
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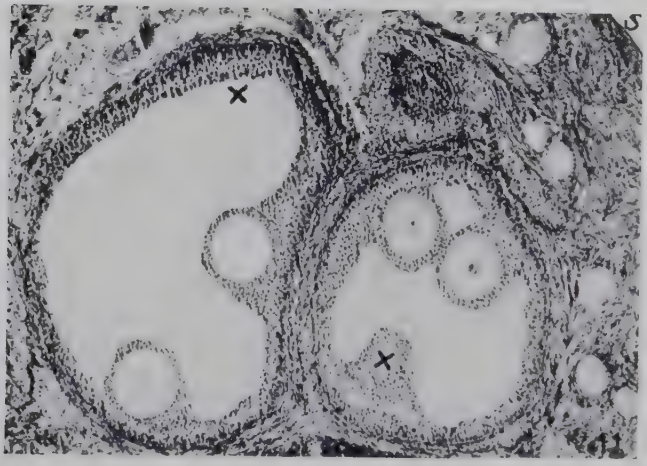
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THE RESPONSE OF THE GONADS OF IMMATURE PIGEONS TO VARIOUS GONADOTROPIC HORMONES [1]

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Riddle ('31) has shown that special advantage attaches to the employment of the response of the testis of the immature pigeon or ring dove as a test object for qualitative or quantitative measurement of the anterior pituitary gonadotropic hormone, toward which it is uniquely sensitive. He has also shown the failure of response of this test object to the material found in the urine of pregnant women, regardless of the dose level employed, and has thus furnished a valuable qualitative difference between this particular urinary hormone and the pituitary gonadotropic hormone.

It has already been shown, however, that the above difference cannot be generalized as a difference between urinary and glandular gonadotropic hormones. With Ferguson and Riddle, we were able to demonstrate a marked response of the dove's testis to the material found in the urine of a man suffering from embryonal carcinoma of the testis (Evans, Simpson, Austin and Ferguson, '33).

The urinary gonadotropic hormones thus appear to differ among themselves, and it would seem important to investigate the several kinds of gonadotropic substances now known with reference to their capacity to stimulate growth of the immature avian testis as compared with their effects on the ovaries of immature rats. The present paper is devoted to this end.

[1] Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation of New York City. The authors desire to record their appreciation of the invaluable aid of Donald H. Wonder in the preparation and standardization of the hormones employed in these studies.

METHODS

The Silver King strain of squabs obtained from a single pigeonry was used in these experiments. As it might reasonably be expected that differences would be found between birds from single egg as contrasted with two egg nests, the squabs employed were selected from two egg nests. Injections were begun when the squabs were 30 to 33 days old. Three or four squabs were included in each experimental group. They were injected 4 consecutive days intramuscularly (pectoral muscle), with a total of 4 cc. of an aqueous solution of the gonad stimulant to be tested. Autopsy was performed 96 hours after onset of injection.[2]

The minimal effective dose in the rat (1 R.U.) is used as a standard in the discussion of dosages injected. The unitage of each substance was determined by subcutaneous injection of normal female rats 23 to 26 days of age, once daily for 3 consecutive days, followed by sacrifice after 96 hours. The minimal effective dose or unit chosen was the amount necessary to stimulate a single corpus in at least one ovary of one animal of the group of three test rats.

To serve as a norm for the avian gonadal weights untreated pigeons 34 to 37 days old were sacrificed and the ovary (left) or the two testes were weighed. Weights from thirty-nine squabs are included in table 1. It will be seen that the ovary weighed between 29 and 55 mg., averaging 40 mg., and that the two testes varied in weight between 11 and 38 mg., averaging 23 mg.

PART I

RESPONSE OF AVIAN GONADS TO HYPOPHYSEAL GONADOTROPIC HORMONE

As the age and strain of bird used, and also the conditions of injection were different than those used by other workers, it seemed important first to establish the sensitiveness of the

[2] Food was withdrawn 24 hours before autopsy, as the birds were used to test simultaneously for the presence of lactogenic and thyrotropic hormones in the preparations.

gonadal response to hypophyseal preparations. Acetone desiccated whole pituitaries of sheep and pigs were employed as starting material for the preparations used in these tests. The particular preparation cited in table 2 was a flavianate (Evans, Meyer and Simpson, '33). It had been standardized

TABLE 1

Weights of gonads of normal untreated Silver King pigeons 34 to 37 days of age

FEMALES		MALES	
Body weight	Weight of ovary	Body weight	Weight of testes
gm.	*mg.*	*gm.*	*mg.*
475	39	360	11
430	35	430	27
450	42	440	21
436	55	430	30
380	31	392	16
410	29	436	38
336	31	366	12
413	30	444	22
370	40	472	18
480	50	550	27
410	53	360	25
380	46	450	24
450	34	492	16
460	42	565	26
413	30	368	21
410	53	472	18
370	40	530	27
480	50	430	36
440	40	412	15
		464	31
Average 421	Average 40	Average 443	Average 23

in rats by administration in four-dose levels. The lowest dose tested, 2.7 mg., was somewhat above one rat unit (1 R.U.) as all three animals responded and the average weight of the ovaries was 40 mg. At ten times this dose, the hypophyseal hormone stimulated rat ovaries averaging 81 mg. This preparation was administered to squabs at approximately the same levels used in rats. The level which corresponded to approxi-

mately 1 R.U. produced testes in the pigeons weighing about six times those of untreated controls. It is therefore apparent that the immature testis of the pigeons used in these studies was a much more sensitive test object for the pituitary gonadotropic hormone than the immature rat's ovary. The pigeon's ovary, on the other hand, only began to increase in weight appreciably when 10 R.U. were administered.

TABLE 2

Response of immature pigeon gonads to administration of gonadotropic hormone from the anterior pituitary

TREATMENT	IMMATURE PIGEONS			IMMATURE RATS	
	Dose	Weight of ovary	Weight of testes	Dose	Weight of ovaries
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Hypophyseal gonadotropic hormone, flavinate, pig (W1897)	36	125	124	27.3	90
		90			70
					76
	18	65	185	13.6	40
			90		43
			205		70
				6.8	34
					20
					27
	3.6	62	64	2.7	38
			135		29
			75		50

PART II

THE NON-RESPONSIVENESS OF THE IMMATURE PIGEON'S GONADS TO COMBINATIONS OF HYPOPHYSEAL SYNERGIST AND PREGNANCY-PROLAN

In reflecting on the great sensitiveness of avian testes to hypophyseal hormone and their complete lack of response to pregnancy-prolan, one is tempted to compare the situation with that found in hypophysectomized animals (Reichert, Pencharz, Simpson, Meyer and Evans, '32) where pregnancy-prolan will not give typical gonadotropic effects, though these are readily secured from pituitary gonadotropic hormone.

There is, however, a substance in the hypophysis [3] which, though possessed of almost negligible gonadotropic action has, when combined in vitro with prolan, typical gonadotropic properties in hypophysectomized animals (Evans, Meyer and Simpson, '32; Evans, Pencharz and Simpson, '34).

Since the pigeons' testes and hypophysectomized rats' ovaries both fail to develop under the influence of pregnancy-prolan alone, it seemed important to test the avian response to prolan in the presence of the synergist. If this combination of prolan and synergist had created a substance identical [4] with, or comparable to the true hypophyseal gonadotropic hormone, it should be detected by the avian testis which is so extraordinarily sensitive to traces of hypophyseal gonadotropic hormone. It might be anticipated that such synergism experiments would be rendered difficult by the traces of gonadotropic hormone which have always contaminated preparations of the synergist. It has been possible to obviate this difficulty, however, by varying the concentration of the synergist, by using preparations of different degrees of purity, and by having control groups of birds treated only with the synergist.

In the first experiments a synergist from the hypophyses of beeves was used. The glands of these animals, though somewhat deficient in true gonadotropic hormone are nevertheless an excellent source of the synergist, as was shown in

[3] The hypophyseal substance involved in this reaction has been provisionally designated by us as the synergist or activator. Though not completely free of gonadotropic properties, the synergist can be obtained in preparations free of growth hormone, also thyrotropic and lactogenic hormones (Evans and Simpson, unpublished data).

[4] Other lines of evidence speak against the conception that the synergist-prolan combination involves a chemical synthesis of the true glandular gonadotropic hormone. For example, a mixture of synergist and prolan was digested with trypsin which destroyed prolan almost completely, but left the synergist unaffected (Evans, Simpson and Austin, unpublished data).

Synergist and prolan combined in vitro	Ovaries 139 mg. (control)
Combination maintained 8 hours at 37°C. at pH 8.5	Ovaries 140 mg. (control)
Digest of combination with trypsin 8 hours at 37°C. at pH 8.5	Ovaries 59 mg.
Digested combination combined in vitro with prolan	Ovaries 145 mg. (prolan alone 31 mg.)
Digested combination combined in vitro with synergist	Ovaries 39 mg. (synergist alone 33 mg.)

the original experiments (Evans, Meyer and Simpson, '32). The synergic substance from beef hypophysis used in the pigeon was a simple 40 per cent alcohol extract precipitated in 80 per cent alcohol, dried and redissolved in a small volume of water (W1810). The prolan used was an alcoholic precipitate from the urine of pregnant women.[5] The levels of the hypophyseal synergic substance first injected were about ten times greater than those necessary for maximal synergism in the rat. The levels used in rats and pigeons was therefore in approximately the same ratio as were the actual body weights of the rats and birds. The prolan, as will be seen in table 3, was completely ineffective on either ovary or testes of the pigeon. On the other hand, synergist from the beef hypophysis was a stimulant of both the testes and ovary of the pigeon (though ineffective in rats at the highest dose given). The two substances combined in vitro, in the same doses given separately, had almost the same gonadotropic effect, probably slightly less, than the synergist administered alone. Synergism was therefore not evidenced.

A more purified synergist was next tested. The preparation was a trypsin-erepsin digest (Evans, Simpson and Austin, '33) of desiccated pig anterior hypophysis (W1724, table 2). It was relatively low in gonadotropic hormone as indicated by tests on normal immature female rats,[6] but was found to have distinct gonadotropic properties in the bird on both testes and ovary. As in the preceding experiment, prolan alone had no effect on the gonads. The combination had slightly less gonadotropic effect than had the synergist alone, thus confirming the experience of the preceding experiment.

As relatively few males happened to occur among the four birds used in each of the six preceding groups injected,

[5] This alcohol precipitate from urine of pregnant women was kindly furnished us by E. R. Squibb & Son through the courtesy of Dr. J. A. Morrell and by I. G. Elberfeld through the courtesy of Drs. H. Hörlein, W. Schulemann and F. Laqueur.

[6] Two out of three rats had infantile ovaries; one reacted having 42-mg. ovaries, bringing the average, as cited in the table, to 33-mg.

TABLE 3

Gonadotropic action of pregnancy-prolan, hypophyseal synergist and combinations of the two factors on immature pigeons

TREATMENT	IMMATURE PIGEONS				STANDARDIZATION IN IMMATURE RATS		
	Dose	Weight of ovary	Weight of testes	Activation	Dose	Average Weights of ovaries	Activation
	mg.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Hypophyseal synergist 40 per cent alcohol extract beef (W1810)	92	112 70 127 106			27.3 13.6 6.8	25 23 15	
Pregnancy-prolan, alcohol precipitate	67 (49 R.U.)	55	22 37 29		27.0	31	
Combination	92 67	107 40 36	78	*0*	27.3 27.3 13.6 27.3 6.8 27.3	87 66 87	*129* *66* *212*
Hypophyseal synergist trypsin-erepsin digest, pig (W1724)	30.8	55 31 119	89		2.7	33	
Pregnancy-prolan, alcohol precipitate	31.7	25 38	35 39		6.8	34	
Combination	30.8 31.7	51 68 64	69	*0*	2.7 6.8	94	*92*
Hypophyseal synergist trypsin digest, pig (W2050)	2.7	66 46	28 17		2.7	28	
Pregnancy-prolan, alcohol precipitate	6.8	35 24 58	31		6.8	25	
Combination	2.7 6.8	51	28 24 39	*0*	2.7 6.8	93	*165*
Hypophyseal synergist (W2050)	27.3	44	51 58 57		27.3	19	
Pregnancy-prolan	68.2	33 32	28 38		68.2	86	
Combination	27.3 68.2	44 53	25 24	*0*	27.3 68.2	93	*6*

another series of experiments was devised to increase the reliability of these results. A further object was an examination as to whether the failure of activation in the preceding experiments had been due to an unfortunate choice of dose levels. In the rat test for synergism, the dose level is a very important factor. If either component, at the dose given, has too high an inherent gonadotropic action, the result of injecting the combination frequently fails to exceed the additive effects, whereas the same components at lower dose levels may show excellent synergism. A level of synergist was therefore chosen for injection in the pigeons which gave excellent activation in rats when combined with prolan, yet by itself was low or lacking in gonadotropic activity. The preparation used (W2050, table 3) was a trypsin digest of pig pituitaries. The results secured from injecting rats and pigeons with this dose level of synergist, given alone and in combination with prolan are shown in table 3 (W2050). As can be seen the low level of synergist was lacking in gonadotropic activity in the bird as well as in the rat. The prolan also, as was expected, had no effect on either testis or ovary of the bird. The combination also left completely infantile gonads in the injected birds. It will be noted that this same level gave 165 per cent activation in rats. At a higher level, ten times the preceding dose level, the synergist began to cause the development of the bird testes, though the ovary remained infantile. The high dose of prolan had no effect on either testis or ovary of the bird. The combination had even less gonadotropic effect on the pigeons than did the synergist alone—an experience encountered in the two preceding experiments. These high levels of synergist and prolan were both too pronounced in their gonadotropic activity in immature rats to enable the activation phenomenon to be shown.

The work just recounted shows that a hypophyseal extract can be prepared which at the level giving typical, excellent synergic effects when combined with prolan in immature rats, is by itself devoid of gonadotropic effects in this species.

Synergically active levels of such preparations may even be devoid of gonadotropic effect on the more sensitive testis of the immature male pigeon. Though such combinations of synergist and prolan have markedly increased gonadotropic effects in immature rats, no such augmentation was observed in immature male pigeons. At higher levels also, levels at which the hypophyseal synergist administered alone began to evidence gonadotropic effects on the immature pigeon testis, the combination with prolan did not give increased effects. In fact, the gonadotropic effect of the synergist on the bird's testis was decreased in each instance by its combination with prolan.

The results may perhaps be interpreted as sustaining the use of the term 'activator' for the hypophyseal constituent of the mixture, for if prolan were the activator, one would have expected it to increase, if only slightly, the gonadotropic properties shown to be present in the hypophyseal constituent. Furthermore, it would appear evident that the combination of any hypophyseal synergist with pregnancy-prolan does not synthesize or in any way create the true pituitary gonadotropic hormone.

PART III

ACTION OF MENOPAUSE-PROLAN ON THE IMMATURE AVIAN TESTIS

It may be regarded as established that pregnancy-prolan does not affect the immature avian testis, though this organ is extraordinarily sensitive to hypophyseal gonadotropic hormone. Evidence is accumulating for the conception of the actual formation of pregnancy-prolan by the chorionic constituents of the placenta. On the other hand, it is difficult to attribute the formation of the gonadotropic substance found in the blood stream and urine after the menopause (menopause-prolan) to any other source than the hypophysis itself. Indeed, other facts, which need not be detailed here,[7] indicate a different chemical makeup and probably different origin for

[7] For example, its effect is augmented by combination with pregnancy-prolan (Leonard and Smith, '34). Further, the ratio of rat and mouse dose are not the same (Hamburger, '33).

menopause- and castrate-prolan as distinguished from pregnancy-prolan, pointing to a hypophyseal origin of the former substances. It was therefore of interest and of some importance to test the action of menopause-prolan on the most sensitive test object known to us for the detection of true hypophyseal gonadotropic hormone, the immature avian testis.

TABLE 4

Lack of response of immature bird gonads to menopause urines of proved gonadotropic efficacy in immature rats

TREATMENT	TOTAL DOSE (EQUIVALENT IN URINE)	IMMATURE PIGEONS		IMMATURE RATS	
		Weight of ovary	Weight of testes	Weight of ovaries	Structure of ovaries
	cc.	*mg.*	*mg.*	*mg.*	
Menopause urine water extract of alcohol precipitate (W1913)	118	62, 36	46, 34		
	99			75, 130, 90	Follicles only, vascular penetration of granulosa (ovulation occasionally)
	59	35	26, 28, 31		
	49.5			21, 22, 35	Few medium large follicles in one animal
Menopause urine water extract of alcohol precipitate (W1912)	118	17, 75, 15	35		
	99			28, 43, 26	Medium and large follicles
	48			48, 19, 25	Large follicles in one animal
Menopause urine water extract of alcohol precipitate (W2354)	137	19, 19	18	30, 39, 41	Many large follicles

As will be seen in table 4, the hormone found in the urine of women past the menopause did not stimulate the testes of immature pigeons, even at doses far greater than those effective in immature rats. In the first case cited in the table (W1913) the product injected was minimally effective on the ovary of the immature rat at a dose equivalent to 49.5 cc. of urine and a dose equivalent to 99 cc. of urine gave 130 mg. ovaries. This is a weight of ovaries almost never, or never,

attained in 96 hours by the injection of pregnancy-prolan and would indicate in itself that the substance in the menopause urine was different from pregnancy-prolan quite aside from qualitative differences in the ovaries stimulated by it. It behaved like prolan, however, in that it was ineffective in the bird at about three times the dose effective in rats (equivalent of 118 cc. of urine). The weights of testes and ovary of two pigeons were slightly beyond the limits observed in the controls cited in table 1, but histologically very little or no change was detectable in these organs. The second menopause urine tested in birds (W1912) also had no effect on three out of four birds; the weight of the ovary of the fourth bird was, however, beyond the limits of variation of controls cited. The third menopause urine, of proven efficacy in immature rodents, was also ineffective in birds, though given in a dose equivalent to 137 cc. of urine. Still higher doses should be administered [8] to make it conclusive that the pigeon's testis and ovary will not react at any dose to menopause urinary hormone, but at least it is clear that the immature pigeon's testis (and ovary) is not so sensitive as the immature rat ovary to this material.

In summarizing, it is evident that the testis of the immature pigeon, while responding sensitively to low levels of pituitary gonadotropic hormone, exhibited no response to three samples of menopause-prolan at levels several times those necessary to stimulate detectable effects in the immature rat ovary, indicating chemical dissimilarity in the hormones.

PART IV

ACTION OF URINE FROM A CASE OF EMBRYONAL CARCINOMA OF THE TESTIS

Aside from the menopause urine, one other type of urine containing gonadotropic hormone was tested, that from a case

[8] It was not found possible to use acetone (60 per cent) and ammonia (4 per cent) to further concentrate the material present in menopause urines or that in the urine from the case of embryonal carcinoma of the testes cited below. This can readily be accomplished by the treatment of pregnancy-prolan with alcohol and ammonia (Evans, Meyer and Simpson, '33). This process which appears to destroy menopause prolan, yet concentrates the hormone in pregnancy-prolan, would indicate a chemical difference between these hormones.

of embryonal carcinoma of the testis in which metastasis had occurred. The urine from this patient (table 5) behaved like menopause urine in that it was effective in rats, but had no effect on the squab gonad. When urine from this case was first tested at a dose equivalent to 7.5 cc. it produced ovulation in immature rats yet had no effect on immature pigeon

TABLE 5

Lack of response of immature pigeon gonads to urine from a case of embryonal carcinoma of the human testis

TREATMENT	TOTAL DOSE (EQUIVALENT IN URINE)	IMMATURE PIGEONS		IMMATURE RATS	
		Weight of ovary	Weight of testes	Weight of ovaries	Structure of ovaries
	cc.	*mg.*	*mg.*	*mg.*	
Urine from case of embryonal carcinoma of the testis.[1]	44	29, 31	18		
	30			28, 41, 60	Large follicles and few corpora
Water extract of alcohol precipitate (W1915)	22	41, 21	25, 32	20, 48, 27	Corpora and large follicles, ovulation
	15			26, 31, 37 47, 42, 29	Corpora
	7.5			20, 16, 32	Corpora, ovulation
Same case 3 months later (W2358)	70	40	36, 15, 31	62, 180	Corpora, large follicles
	20			77, 75, 65	Corpora, large follicles, cysts and blood points
	10			85, 58, 38	Corpora, large follicles, cysts and blood points
	2			27, 30, 35	Few corpora, ovulation

[1] This urine was kindly made available to us through the courtesy of Drs. Frank Hinman and Tracy Powell of the genitourinary department of the University of California Medical School. The patient had had one testis removed previously. At the time urine was first collected metastases were present. It will be noted that the gonadotropic unitage had tripled during the 3-month interval between the first and second tests of the urine.

testis (or ovary) at a dose equivalent to 40 cc., i.e., over five times the effective rat dose. Urine from the same patient 3 months later, when metastasis was more advanced, had tripled in potency as judged by the rat, but it was still ineffective in squabs even at thirty-five times the minimal rat dose.

In its incapacity to stimulate growth of the squab's gonads this material resembled pregnancy-prolan. It is evident that the urinary hormone in this testicular neoplasm does not resemble that in the case reported with Ferguson for the latter substance was a potent stimulant of the growth of the immature dove's testis (Evans, Simpson, Austin and Ferguson, '33).

PART V

EFFECT ON THE GONADS OF THE IMMATURE PIGEON OF THE GONADOTROPIC HORMONE IN THE BLOOD STREAM AND TISSUES OF MARES EARLY IN PREGNANCY

The original discovery of Cole and Hart ('30) and the comparisons made by Evans, Meyer and Simpson ('33), established the high potency of the gonadotropic substance found in the blood stream of pregnant mares. In fact, this work amply established the fact that this substance, as measused by its effect on the rat, is the most potent gonad-stimulant known to us. Fifty units of this hormone can in the 96-hour period produce ovaries in immature rats approaching 400 mg., an effect far greater than that obtained with an identical unitage of anterior pituitary hormone.[9]

The origin of the equine hormone, as is the case with that found in the blood and urine in human pregnancy, is under dispute. Catchpole and Lyons ('34) have advanced reasons for assigning it an extrahypophyseal (i.e., placental) origin. It was consequently interesting to employ the testes of immature pigeons in comparing the effect of this substance with that of hormone derived from the hypophysis itself.

[9] Illustrating the slighter effect of increased dosage with hypophyseal hormone is the following: 5 mg. of a water-soluble fraction, obtained by extracting sheep glands with 50 per cent aqueous pyridine according to the method of Fevold et al., ('31) was given to immature rats in a total dose 1.36 mg. per rat, giving on the average 37-mg. ovaries after 96 hours. The dose was therefore only slightly more than 1 R.U. As this was a very soluble fraction, it was possible to administer it in fifty times this dose (68 mg.), at which level it gave on the average only 154-mg. ovaries.

The hormone cited in table 6 was secured by extraction of the endometrium of mares in the early stages of pregnancy delimited by colt lengths of from 2 to 6 inches. The tissue

TABLE 6

Response of immature pigeon gonads to administration of gonadotropic hormone from pregnant mare's endometrium

DOSE	IMMATURE PIGEONS		IMMATURE RATS	
	Weight of ovary	Weight of testes	Dose	Weight of ovaries
mg.	*mg.*	*mg.*	*mg.*	*mg.*
13.4 (134 R.U.)	47	275		
	132	110		
	102	89		
		135		
		160		
6.7 (67 R.U.)	101	133	5.0	385
	88	94		206
	55	140		238
	54	125		347
				290
				158
1.34 (13 R.U.)	61	37	1.0	118
	68	55		98
	21			162
	37			128
	42			340
	62			225
0.67 (6.7 R.U.)	46	50		
		26		
		56		
0.13 (1.3 R.U.)	60	34	0.10	28
	35	19		27
				30

was ground and extracted three times with ten volumes of 60 per cent acetone and 4 per cent of ammonia. The extract was then precipitated in 90 per cent acetone on addition of a few drops of glacial acetic acid. The extracts were administered at three-dose levels to immature rats, the lowest level

being that which could produce detectable results (1 R.U.). As can be seen in table 6, at fifty times this level (5 mg. or 50 R.U.) the hormone stimulated very heavy ovaries, averaging 271 mg. The same preparation was administered to the 1-month-old Silver King squabs at five-dose levels, covering and exceeding the dosage range tested in rats. Detectable effects on the birds' testes were not secured at the 1.3 R.U. level. At both the 6.7 and 13 R.U. levels the response of the testes were still within the range of what could be designated as 1 pigeon unit, since the testis of one of the two male squabs in the group did not react even at 13 R.U. At the 67 R.U. level a six-fold increase in weight of the testes was observed. While beginning response of the pigeon's testis was detected at about 6.7 R.U. the pigeon's ovary did not show any response until the 13 R.U. level was reached. At ten times this dosage the ovaries attained a weight of 132 mg.—a weight about three times that of the ovary of control pigeons.

From these results it is clear that even though the gonadotropic hormone found in the blood stream and placenta of the pregnant mare is more potent in its effects on the ovaries of immature mammals than is hypophyseal gonadotropic hormone, it does not act with comparable potency on the immature pigeon's testis.

CONCLUSIONS

1. Confirmation has been secured of the demonstration by Riddle that the testis of immature pigeons is peculiarly sensitive to the gonadotropic hormone of the anterior hypophysis and is insensitive to the material in the urine of pregnant women.

2. The above facts, however, cannot be generalized as representing a characteristic difference in two general types of gonadotropic hormone, i.e., those of pituitary and those of urinary origin, respectively, because a urinary hormone has been described in a case of testicular tumor giving a maximal acceleration of the growth of the squab's testis.

3. Nor can it be claimed that the immature avian testis is a sensitive test object for the most potent gonadotropic hormone known to us, that present in the blood stream in mares early in pregnancy. Doses of the equine hormone, which gave minimal but definite effects in the rat have no recognizable effect on the immature avian testis.

4. The material present in the urine of women after the menopause represents another gonadotropic hormone with effects more easily detected by changes in the immature rat's ovary than by the immature pigeon's testis. This was also true of another case of embryonal carcinoma of the testis described in this paper.

5. The squab's testis does not show the phenomenon of augmentation or synergism which is evidenced by increased ovarian weights when immature rats are treated with certain anterior pituitary extracts (synergist) added in vitro to pregnancy-prolan. Since the squab's testis is peculiarly sensitive to the gonadotropic hormone secured from the anterior hypophysis, it is apparent that the mixture of prolan and hypophyseal synergist does not constitute a substance identical with the anterior hypophyseal hormone.

6. It would seem apparent from the foregoing that the effect of various gonadotropic hormones on the immature pigeon's testis is peculiar for each kind, or source, of gonadotropic substance and by no means parallels the effect of the same substance on the immature rat's ovary.

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REDUCTION OF THE THYMUS BY GONADOTROPIC HORMONE [1]

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The meagerness of well-established results from the numerous efforts to determine the function of the thymus is too well known for further comment. (See, for instance, Trendelenburg's recent account, '34.) It is not too much to state that no investigator has as yet been able to secure specific effects from ablation of the thymus or from efforts to experimentally imitate increased thymic function. Security in the conception of the thymus as an organ of internal secretion and light on its role may, however, come from a study of the effect of other internal secretions on the thymus. The present study aims to report one such effect—one resulting from injection of a gonadotropic hormone. Chronic injection of this hormone led to a striking reduction of thymic substance sometimes resulting in almost complete disappearance of the gland—an effect which, as will be disclosed, is probably to be attributed to the intermediation of male or female sex hormone.

The years of painstaking quantitative studies devoted by August Hammar to normal and altered thymic structure established the striking and sensitive dependence of the thymus upon the maintenance of normal body weight and satisfactory general nutrition, demonstrating as it did the

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rapid recession of the thymus in practically all disturbances of growth or nutrition. This relationship is so securely established that any claims for experimental contrivances which electively reduce the thymus cannot be accepted if impairment of body weight or of growth or other adverse effects are demonstrable. The experimental procedures herein reported were not associated with loss of body substance; they were, indeed, associated with slight gains in adults or with moderate or marked gains of weight in immature animals.

The gonadotropic hormone used in these experiments is the most potent gonadotropic substance known to us, namely, that found in the placenta or in the blood stream of mares early in pregnancy (Cole and Hart, '30; Evans, Meyer and Simpson, '33; Catchpole and Lyons, '34). The particular product used was an extract of mare's placenta and uterus at the appropriate early period of pregnancy.

CHRONIC TREATMENT OF NORMAL ANIMALS WITH EQUINE GONADOTROPIC HORMONE

A. Immature females

Female rats, 25 days of age, were injected once daily subcutaneously with 50 R.U. of gonadotropic hormone for 26 to 65 days. Normal uninjected littermate controls were sacrificed at the end of this injection period along with the injected animals. A report has already been made of the striking hypertrophy of the ovaries and of the anterior hypophysis which characterized these injected animals.[2] Only the effect on the thymus is considered here. As will be seen in table 1 (S25), the thymus of the controls weighed between 202 and 535 mg., averaging 264 mg., while the thymus of their littermates treated chronically with potent gonadotropic hormone were reduced to weights of only 8 to 83 mg., average 46 mg.

[2] The original purpose for which chronic injections of this gonadotropic hormone was undertaken was to determine the effect on the ovary. The effects on the ovaries and hypophyses have been reported elsewhere (Evans, Simpson, and McQueen-Williams, '34; Evans and Simpson, '34).

TABLE 1

Response of thymus of mature and immature male and female rats to chronic injection of the gonadotropic hormone secured from pregnant mares

TYPE OF ANIMAL	AGE	EQUINE GONADOTROPIC HORMONE 50 R. U. DAILY			CONTROL	
		Duration of injection	Increase in body weight	Thymus	Increase in body weight	Thymus
	days	*days*	*gm.*	*mg.*	*gm.*	*mg.*
Females immature (S25)	25	65	130	62	139	202
	25	65	100	54	126	280
	25	65	98	35	164	260
	25	65	112	49	124	245
	25	65	129	243 [1]	96	127
	25	65	106	60	120	300
	25	65	118	35	214	220
	25	65	111	44	146	231
	25	65	94	25	134	204
	25	65	98	8	140	256
	25	61	142	53	57	535
	25	26	98	83	141	305
Average			111	46	133	264
Females mature (S27)	177	65	46	23		146
	177	65	4	49		97
	177	65	— 8 [2]	57		129
	176	65	28	11		86
	177	65	3	65		174
	177	65	4	40		139
	177	65	— 4 [2]	32	Plateaued	106
	177	65	— 4 [2]	26		132
	177	65	18	53		82
	196	65	— 6 [2]	10		
	195	50	6	10		
	196	30	9	10		186
	196	20	0	10		
Average			7	30		127
Males immature (S24)	25	65	170	27	250	191
	25	65	134	47	304	270
	25	65	162	85	278	272
	25	65	184	63	334	350
	25	65	168	60	350	265
	25	65	168	80	224	285
	25	20	100	530	310	400
Average			155	60	292	290
Males mature (S26)	122	65	— 25 [2]	27		196
	120	65	13	36		260
	122	65	20	39		347
	120	65	18	42	Plateaued	290
	133	65	10	61		290
	121	65	12	17		400
	120	147	— 2 [2]	35		204
	120	30	0	207		
Average			6	37		284

[1] This animal also did not show the characteristic hypophyseal hypertrophy seen in other members of this group. The hypophysis weighed 12.5 mg. Those of others in the group 26, 21, 26, 26 mg.

[2] No animal is included in the table which lost weight during the last 10 to 20 days of injection. Animals are also excluded known to be sick, e.g., wheezy rats, rats injured in fighting, etc.

B. Mature females

Mature females, 6 to 6½ months of age, were treated once daily subcutaneously with an identical amount of the same preparation injected into the immature females. The adult females were also sacrificed after 20 to 65 daily injections were given. Here also, associated with the massive hypertrophy of the ovaries [3] and of the pituitary, an astonishing reduction of the thymus was encountered; at times the vestiges of thymus present were too small to give security in dissection for weighing (those cases noted in the table where the weight was 10 mg. or less). Even the thymi of the females treated for the shorter periods, 20 to 30 days, showed extreme atrophy (average 30 compared with a 127 mg. average in the controls). See table 1, S27.

C. Immature males

Immature males, 25 days of age, were similarly injected for 20 to 65 days. With the single exception of the animal injected only for 20 days, all animals showed thymi reduced to one-fifth the weight of normal uninjected animals of the same sex and age, 60 compared with 290 mg. See table 1, S24.

D. Mature males

A group of adult males, 4 to 4½ months old, was also treated for varying periods, 30 to 147 days. In this group the atrophy of the thymus was extreme, except in one animal, sacrificed after only 30 days' injection. The average thymus weight was 37 mg. compared with 284 mg. average in control males. See table 1, S26.

It is to be pointed out that at time of sacrifice all animals included in the tables were in excellent nutritive condition. All immature animals had gained weight during the injection

[3] These ovaries as can be seen by consulting the paper dealing with this matter (Evans, Simpson and McQueen-Williams, '34) sometimes attained a weight of 2 to 3 gm. The original object of this experiment was to see if the ovary became exhausted by long continued injection with the equine hormone. It was found, however, that they weighed between 1 and 2 gm. after more than 2 months' injection.

period.[4] All adults had either gained slowly or were in growth stasis. None were included that had lost any weight during the last 10 to 20 days of the injection period. There is no reason, therefore, for referring the thymic atrophy to malcondition of the animal. Instances were noted such as a rat injected 30 days in which the thymus weighed more than that of any other animal in the group, namely, 207 mg. yet during the full period of administration no body growth occurred in the animal, whereas others in the group gained as much as 20 gm. yet the thymus was completely atrophic.

INJECTION OF GONADECTOMIZED RATS WITH THE EQUINE GONADOTROPIC HORMONE

The following experiments show that, regardless of the sex employed, gonadectomy prevents this experimental reduction of the thymus by gonadotropic hormone.

A. Females

Females 38 days of age were castrated and immediately thereafter injected with 125 R.U. of pregnant mare gonadotropic hormone daily for 20 days (table 2). At the end of this period, they were sacrificed. Normal uninjected littermate controls were maintained and sacrificed at the same time. The thymi of the untreated controls average 393 mg. in weight, those of the ovariectomized animals treated with the hormone averaged 453 mg. The gonadotropic hormone, therefore, exerted no clear degenerative influence on the thymus in the absence of the ovaries.[5] A group of normal females of the same age, many of them littermates to the above, were injected with the same hormone during the 20-day period, and their

[4] Comparison with normal uninjected animals shows, however, that a definite although not serious interference with growth resulted from administration of these substances to the males. The administration of gonadotropic hormone from other sources (anterior hypophyses, urine of pregnancy) will ultimately be necessary before we can refer the slower growth seen in the male to the effects of the gonadotropic hormone itself.

[5] Gonadectomy also prevented the hypertrophy of the anterior hypophysis which resulted from the administration of the same gonadotropic hormone (Evans and Simpson, '34).

thymi showed significant decrease in weight (103 mg.) showing that the dose and period of injection were adequate.

TABLE 2

Response of the thymus of male and female castrated rats to the gonadotropic hormone from pregnant mares

TREATMENT	AGE	DURATION OF INJECTION	INJECTED				CONTROL			
			Castrate		Normal		Normal		Castrate	
			Increase in body weight	Thymus	Increase in body weight	Thymus	Increase in body weight	Thymus	Increase in body weight	Thymus
	days	*days*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
Males			50	455	27	40			68	390
equine			72	440	80	163			96	410
hormone			76	426	64	157			99	410
12.5 R.U.	39–45	20	54	507	70	187			52	310
daily			48	445	12	10			62	440
(W1238)			44	572	40	52			44	224
Average			57	474	49	101			70	364
Males			108	415			92	470	146	320
equine			90	410			126	395	146	375
hormone			102	440			124	660	156	550
25 R.U.	30	30	98	470			120	510	140	485
daily			102	645			108	490	158	320
(W1381)			128	460			116	440	152	355
							91	250		
							124	540		
Average			104	473			112	470	150	401
Females			73	400	26	111			60	435
equine			102	495	40	39			76	530
hormone			78	720	62	170			48	300
12.5 R.U.	38	20	56	310	62	115			62	400
daily			76	250	48	126			58	515
(W1238)			68	545	46	58			42	176
Average			76	453	47	103			58	393

B. Males

A group of six young males (39 to 45 days of age), were castrated and injected at once with the same unitage of gonadotropic hormone used in the preceding experiments. Injections were continued 20 days. As in the case of females, the thymus did not show the characteristic atrophy after

castration, the average weight being 474 mg. as compared to 364 mg. in the normal males of the same age. The normal littermates of these animals receiving the same dose for the same period of time had thymi reduced to a fourth of this figure (101 mg.).

In the second group of six castrate males, injections were also begun immediately after operation, but continued 30 days, the dosage being 25 R.U. daily. Similar results were obtained, i.e., no reduction of the thymus; the thymi averaged 473 mg., those of their normal uninjected castrate controls, 470 mg., those of their normal uninjected controls, 401. mg.

TREATMENT WITH GONADOTROPIC HORMONE OF MALES STERILE DUE TO CRYPTORCHIDISM OR VITAMIN E DEFICIENCY

An attempt was next made to determine which tissue of the testicles, the seminiferous epithelium or the Leydi gtissue, is responsible for the gonadal secretion stimulated by the gonadotropic hormone that causes the thymic atrophy. For this reason animals were selected in which spermatogenic tissue had been destroyed without loss of Leydig tissue. Such animals were obtained in two ways, 1) by rendering them cryptorchid, and, 2) by maintaining them for a long period on a diet deficient in vitamin E. These two types of sterile animals were then treated as above with equine gonadotropic hormone.

Cryptorchid males

The males were cryptorchidized by anchoring the testicles to the lateral body wall in a subphenic position with two or more ligatures which passed through the whole tunica albuginea. A period of 21 days was allowed to elapse between operation and injection in order to allow extensive degeneration of the seminiferous epithelium. After this interval had elapsed, one testicle was removed and sectioned for information as to its condition. Ripe germ cells were no longer present in the testes; in fact, few cells other than spermatogonia and Sertoli cells remained in the tubules. Leydig tissue had suf-

fered no decrease in amount. As is well known, sex interest and the seminal vesicles of cryptorchidized animals remain normal—facts which have been brought into relation with the persistence of the interstitial tissue of the testes.

The animals were now injected with 50 R.U. daily of the same pregnant mare gonadotropic hormone used in the preceding studies. Injections were continued for 55 days. At autopsy the marked enlargement of the seminal vesicles and hypertrophy of the intertubular strands of Leydig cells portrayed clear effects of the gonadotropic hormone on this portion of the testis. The testicular tubular epithelium, however, doubtless because of the coelomic location of the testis, had not been stimulated. Atrophy was even more complete than at onset of injection. There was virtually an absence of germinal tissue.

Coincident with hypertrohpy of the Leydig tissue and the accessory organs of reproduction, there was a definite reduction of the thymus (table 3). The thymic atrophy was, in fact, as marked in three of the five animals as that secured in normal males by treatment with gonadotropic hormone. The results were therefore in marked contrast to those obtained in the injected castrate males, but in agreement with the behavior of normal males.

Vitamin E deficient males

A further check on the striking results secured by the treatment of cryptorchid males was obtained by the employment of males in which the seminiferous epithelium had been gravely injured or destroyed by the second contrivance, the elimination of vitamin E from the diet.

Nine males were placed from the date of weaning on a dietary regimen devoid of vitamin E.[6] When about 7 months of age, at a time when, according to our previous experience,

[6] Diet contained commercial casein, 27.0; cornstarch (cooked), 35.0; lard, 22.0; brewers' yeast, 10.0; salts (E. V. McCollum and N. Simmonds, J. Biol. Chem., vol. 33, p. 63, 1918), 4.0; cod liver oil, 2.0. This diet is mixed together (except for the cod liver oil) and allowed to stand 2 weeks before feeding. The cod liver oil is added the day of feeding.

the degeneration of the tubular epithelium should be almost complete, the left testis was removed from each animal, in order to establish its condition. The testes proved to be

TABLE 3

Effect of the gonadotropic hormone from pregnant mares on the thymus of male rats sterilized by dietary or operative means, yet possessing normal interstitial tissue

TYPE OF ANIMAL	AGE	EQUINE GONADOTROPIC HORMONE 50 R.U. daily			CONTROL	
		Duration of injection	Increase in body weight	Thymus	Increase in body weight	Thymus
	days	*days*	*gm.*	*mg.*	*gm.*	*mg.*
Cryptorchid adult males (S21)	188	55	32	10	52	249
	176	15	7	85	6	195
	174	55	34	73	34	162
	185	55	68	305	18	156
	173	55	— 2	80	68	167
	185	55	44	218	56	143
					38	185
Average			31	128	39	180
Males reared and maintained on diet deficient in vitamin E (S22)	148	25	14	64	0	130
	216	25	— 6	52	— 8	160
	216	25	6	72	— 19	100
	216	25	16	85	6	163
	216	25	— 6	32	— 18	91
	216	25	8	10	10	129
	148	25	18	87	26	155
	149	35	— 6	60		
	148	35	6	85		
Average			5	61	0	132
Males reared and maintained on diet deficient in vitamin E, supplemented by wheat germ oil (S23)	215	25	0	83	36	110
	215	25	24	27	28	195
	215	25	24	154	26	141
	215	25	20	137	13	214
	215	25	8	83		
	216	25	26	10		
	216	25	26	10		
Average			18	72	26	165

highly degenerate. The tubules were lined by Sertoli cells and spermatogonia, with a few, frequently enlarged spermatocytes. So-called giant cells were present in the lumen. In

some of the animals an occasional tubule contained spermatids or even a few spermatozoa. The few sperm found in the testicular tubules or epididymis were usually deformed and non-motile. Interstitial tissue was approximately normal in amount. As is well known (Evans and Burr, '27), these animals resemble cryptorchidized males in possessing, while sterile, normal accessory organs of reproduction and a normal libido sexualis. Though these animals mated occasionally with estrous females, no litters resulted.

These animals, whose sterility had been proved both by functional tests and by histological examination of the testis and epididymis, were then injected for periods of 25 to 35 days with equine gonadotropic hormone. At autopsy it was found that the thymus was reduced to about half the weight of that of the control uninjected animals on the identical diet (72 mg. vs. 165 mg.) (table 3).

Another group of males [7] sterilized through lack of vitamin E was handled exactly as was the above group, except that coincidentally with the administration of gonadotropic hormone abundant vitamin E was fed these animals (12 drops of wheat germ oil daily). The vitamin E supplemented by the gonadotropic hormone did not, however, provoke a regrowth of the seminiferous epithelium during the 20 days of the experiment. At autopsy, the testes of these animals, just as in the group receiving the gonadotropic hormone but not vitamin E, consisted of atrophic tubules and hypertrophied Leydig tissue. The thymi of these animals just as those in the preceding group were reduced to half, or less than half of their previous weight (72 as compared with 165 mg.). The injected animals and their controls both increased in body weight slightly during this period, due to the improved condition of the animals on addition of vitamin E to the diet.

[7] This group of males was originally chosen for another use. It was known that administration of high vitamin E alone would not (or rarely at least) restore testes of males rendered sterile due to deficiency of E. Here it was hoped that simultaneous presence of vitamin E and a potent gonadotropic hormone would accelerate the regeneration of the seminiferous epithelium. No accelerated regeneration was demonstrable in these experiments, but it is possible that 20 days was inadequate time for repair.

TREATMENT OF GONADECTOMIZED MALE RATS WITH FEMALE SEX HORMONES

The experiments heretofore detailed led us naturally to the conviction that the sex hormones, male or female, were directly responsible for the reduction of the thymus which followed administration of gonadotropic hormone. In this way an explanation was afforded the thymus reduction which occurred when cryptorchid or vitamin E deficient males were treated with gonadotropic hormone, but did not occur on similar injection of gonadectomized animals.

A direct test of this hypothesis was attempted by treatment of animals with an estrogenic hormone, amniotin [8] and with ovarian implants containing both the estrus promoting substance and lutein hormone. Males, both castrate and normal, were injected with these substances alone, or coincidentally received gonadotropic hormone. The amniotin was given in doses of 37.5 or 40 R.U. daily subcutaneously. Mature rat ovaries were used as implants, one and one-half glands being given each 4 days. The ovaries were minced sufficiently to inject into the muscle through a coarse cannula.

As can be seen in table 4, the female sex hormone, amniotin, administered alone had no effect on the thymus of the castrate male, but when given simultaneously with the gonadotropic hormone had a definite thymus reducing action. Its effect was, however, not as extensive as was secured with treatment of normal animals with gonadotropic hormone. Similarly, after ovarian grafts alone, the thymus of the castrates remained normal; after combination with gonadotropic hormone, there was a slight reduction.

It is not unlikely that the administration of gonadotropic hormones to normal animals produces a greater total supply, and it would appear certain that it produces a more continuous supply of the male and female sex hormone than can be imitated by direct administration of these hormones. It would appear probable that more concentrated, frequent and pro-

[8] The amniotin used was kindly furnished by E. R. Squibb & Son through the courtesy of Dr. J. A. Morrell.

TABLE 4

Effect of the gonadotropic hormone from pregnant mares on the thymus of castrate male rats receiving female sex hormones

TREATMENT (1)	AGE	DURATION OF INJECTION	CASTRATE MALES		NORMAL MALES	
			Increase in body weight	Thymus	Increase in body weight	Thymus
	days	*days*	*gm.*	*mg.*	*gm.*	*mg.*
Equine hormone 12.5 R.U. daily (W1238)			20	180	27	40
			50	455	80	163
			48	445	64	157
			72	440	70	187
	39–45	20	76	426	12	10
			54	507	40	52
			44	572		
Average			52	433	49	101
Equine hormone 25 R.U. daily (W1381)			108	415		
			90	410		
			102	440		
	30	30	98	470		
			102	645		
			128	460		
Average			105	473		
Amniotin 37.5 R.U. daily (W1383)			92	310		
			78	250		
			66	205		
	30	30	104	290		
			78	405		
			76	140		
Average			82	267		
Equine hormone 12.5 R.U. and amniotin 40 R.U. daily			62	330	58	84
			30	360	24	90
			58	270	82	190
	30	20	26	315	37	106
			56	450	28	164
			74	305		
Average			51	338	46	127
Equine hormone 25 R.U. and 37.5 R.U. daily			50	178		
			52	159		
			58	198		
			60	233		
	30	30	56	191		
			88	305		
Average			61	211		
Ovarian grafts 1.5 minced rat ovaries each fourth day			98	310		
	30	30	106	340		
			104	370		
			108	540		
			94	345		
			116	405		
Average			104	385		
Equine hormone 25 R.U. daily and ovarian grafts 1.5 ovaries each fourth day			78	260		
			70	93		
	30	30	102	395		
			62	205		
			95	430		
			94	420		
Average			84	301		

[1] For weights on control uninjected castrate and normal rats, see controls of 30-day injected groups, table 2.

longed injection of either male or female sex hormone would be effective in reducing the thymus.

SUMMARY

1. Chronic treatment of male or female rats, whether mature or immature, with equine gonadotropic hormone results in atrophy of the thymus.

2. But when the testes or ovaries are removed, gonadotropic treatment does not cause thymic atrophy.

3. The testicular component involved in this reaction appears to be the interstitial tissue and not the germinal epithelium as treatment with the gonadotropic hormone after destruction of the germinal tissue by procedures which leave the interstitial tissue and its dependent accessory organs intact (cryptorchidism or vitamin E deficiency) leads to the same atrophic changes in the thymus as in normal males.

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MOTOR EFFECTS OF SENSORY NERVES EXPERIMENTALLY CONNECTED WITH MUSCLES

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INTRODUCTION

While the fact that a muscle can receive functional innervation from any motor nerve is undisputed, no satisfactory answer has been obtained yet to the question of whether a sensory nerve could form functional motor connections with a muscle. The first experimental attack of the problem dates back to the middle of the last century. Attempts were made to connect proximal stumps of sensory nerves with peripheral stumps of motor nerves. The nervus lingualis was used almost exclusively as the sensory nerve, and the nervus hypoglossus as the motor nerve. After several failures (Bidder, 1842; Gluge and Thiernesse, 1859), successful cases were reported by Philipeaux and Vulpian (1863). Connecting the proximal lingual with the peripheral hypoglossic in dogs they found that, after a few months of recovery, contractions of the tongue muscles could be obtained by stimulating the trunk of the lingual. These results were confirmed by Rosenthal (1864), and the possibility of a functional neurotization of muscles by sensory nerves seemed to be clearly established. Shortly after their first experiments, however, Philipeaux and Vulpian (1863 a) became aware of the fact that the lingual nerve was not purely sensory, but included some components with motor or 'pseudomotor' (Heidenhain, 1883) effects on the tongue; these components were later demonstrated to be contributed to the lingual nerve by the chorda tympani of the facial (Vulpian, 1873; Heidenhain, 1883; van Rijnberk, '15). Thereafter the original experiments on heterogeneous nerve connections were no longer regarded as conclusive.

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The matter remained undecided until Boeke ('17) resumed the experiments of lingual-hypoglossic crossing on the European hedge-hog, in which the lingual nerve is accessible before its union with the chorda tympani, i.e., before receiving its main motor contribution. Boeke's histological examinations made it evident that fibers of the proximal lingual nerve, known for being truly sensory in this animal, had permeated the peripheral hypoglossic stump, reached the tongue muscles and there effected intimate contact with the muscle fibers in form of arborizations which strikingly resembled the muscular endings of motor fibers. Although these observations made it unquestionable that, as far as the morphological aspect is concerned, sensory nerve fibers are capable of uniting with muscle fibers, it was not determined whether the connections were functional in the sense that they would allow excitations to be transmitted from the sensory nerve to the muscle. Since Boeke has purposely omitted to subject his preparations to physiological tests ('17, p. 7) the functional examination remained wanting.

In the meantime other 'heterogeneous' nerve connections have been produced successfully and were shown to be capable of functioning: The peripheral end of a severed motor nerve was found to form functional connections with a spinal cord into which it had been experimentally inserted (P. Weiss, '32). More extensive work was devoted to the establishment of connections between muscles and dorsal roots (P. Weiss, '35): The central end of the dorsal root was disconnected from the spinal cord and inserted into a muscle instead; owing to the integrity of the spinal ganglion, the sensory neurons survived after the operation, and their proximal branches, contained in the dorsal root, established regenerative connections with the muscle fibers. In a number of cases of this sort, it was then found that stimulation applied to the peripheral afferent nerve yielded contractions in the muscle newly attached to the proximal end of that nerve. These cases for the first time produced clear physiological evidence that the transmission of excitations from sensory fibers to

muscle fibers is possible. But one must bear in mind that, as the muscle lies in these experiments on the central end of the sensory nerve, the direction in which the transmission of excitations occurs is the normal direction of afferent excitations, namely, centrad. Therefore, these results do not answer the question whether transmission is possible also in the peripherad direction, such as would be required in order to obtain motor innervation of a muscle from the peripheral end of a sensory nerve. It is a well-established fact that conduction of excitations in a nerve fiber takes place in both directions, the irreciprocity of communication in the nervous system being commonly attributed to some sort of one-way mechanism instituted in the synapses between nerve fibers and end organs and between neurones. In the sensory neurone such a mechanism would prevent excitations from spreading peripherad, back to the sense organs. Hence, it was seriously questioned whether a muscle fiber, substituted for a sensory end organ could really be effectively innervated (Langley and Anderson, '04).

In order to contribute to a solution of this problem, the following experiments were undertaken. Anticipating the results, we may state that decisive proof has been obtained that muscles really can be functionally innervated by peripheral ends of sensory nerve fibers.

MATERIAL AND METHODS

All experiments were performed on half-grown toads (Bufo viridis Laur.), measuring from 4 to 5 cm. from mouth to anus. The animals were operated during the winter months and kept at room temperature. The experiments differ mainly in two points from the earlier ones devoted to the same problem: first, in that special provisions were taken to insure that the nerve to be tested was completely devoid of all motor fibers; and, second, in that the sensory nerve to be used as innervator was not made to connect with a peripheral motor nerve stump, but directly with a muscle.

Mixed nerves can be turned into exclusively sensory nerves by causing the motor fibers to degenerate. In our experiments this was achieved by complete removal of the ventral roots. In order to prevent complications from collateral regeneration or from hypothetic efferent fibers contained in the dorsal roots, the dorsal roots were also severed from the spinal cord. In other words, the spinal ganglia were entirely disconnected from the spinal cord, but left in connection with their peripheral nerves. The operation was performed on the left eighth or ninth spinal nerve, in the way described more extensively in another paper (P. Weiss, '35): The spinal ganglion was luxated from its position in the foramen intervertebrale, and the dorsal and ventral roots were pulled out from the vertebral canal; the spinal ganglion was then dislocated from its original site to a more lateral position on the dorsal wall of the body cavity so that no chance for a reconnection with the spinal cord was left. In some cases, the sympathetic rami communicantes were preserved; in other cases, the sympathetic chain, including the ganglia and the rami, was extirpated in the segment of the operated nerve and in the two adjacent segments. The peripheral nerve, thus made purely sensory, was severed distally, immediately before its anastomosing with the girdle plexus. By the procedure described we obtained a nerve tract, completely isolated, bounded by two cut surfaces, and in which the motor fibers degenerated while the sensory fibers persisted, owing to the integrity of the spinal ganglion included in the tract. There was no anastomosis left between this nerve tract and other nerves.

The muscle on which the innervating capacity of this nerve was to be tested was prepared in the way described in earlier papers (P. Weiss, '31, '35): The muscle (m. gastrocnemius from the right limb) was denervated and transplanted under stretch to the left side of the back of the animal. The cut peripheral end of the isolated sensory nerve was finally inserted into the transplanted muscle, by the same method as outlined in earlier papers.

The operation was performed on thirty-six animals. A great number of them, however, succumbed in the months following the operation to a grave infection by nematodes, so that only nine animals could be examined alive.

On these animals the physiological examination was performed in the following way: In ether anesthesia the site of the transplanted muscle and of its innervating nerve tract was carefully exposed. Then electrical stimuli were applied to various parts of the nerve, and the reactions of the muscle were observed. The stimuli were single induction shocks with 3 volts in the primary circuit. As electrodes two pointed wires of platinum were used, at a distance of about 1 mm. from each other. The electrodes carrying the nerve were slightly lifted, so that contact with the tissues was avoided. The threshold at which muscular contraction could be obtained was determined by varying the coil distance and the angle between the two coils. In order to preclude errors due to an escape of the stimulating current, the following control was always applied: After a series of successful stimulations the nerve was crushed between the electrodes and the muscle and then the stimulations were repeated with the electrodes left in place. The fact that all subsequent stimulations, even with highly increased intensities, failed completely to yield muscular responses, proved that the contractions obtained prior to the crushing had been due to nervous excitations and not to escaping currents. Besides, in all stimulation tests the electrodes were applied to the nerve at the farthest possible distance from the muscle, the distance varying between 15 and 18 mm. In order to eliminate errors that might be introduced by collateral regeneration, the whole preparation under examination, i.e., the muscle and its nerve with the spinal ganglion and the roots attached, was carefully dissected and isolated on a glass plate; then the stimulations were repeated on this isolated preparation outside the body.

The functional examination finished, the muscles were subjected to histological treatment. However, the Bielschowsky silver impregnation, which on all my earlier material had

given satisfactory results, failed consistently on the present material. In view of the excellent work done on the histological side of the problem by Boeke, the omission of histological control in the present work does not seem to be serious.

RESULTS

The results obtained with the nine animals examined are compiled in table 1. Up to 55 days after operation, no stimulation on the nerve has ever become effective on the muscle, even though very strong stimuli (up to c.d. 2 cm.) were applied. All animals kept for 70 days or more after operation, however, gave muscular responses. The observations on the five cases of this latter group were the following:

TABLE 1

PROTOCOL NO.	NERVE USED FOR INNERVATION	CONDITION OF SYMPATHETIC	TIME AFTER OPERATION IN DAYS	MUSCULAR RESPONSE TO NERVE EXCITATION	THRESHOLD INTENSITY (COIL DISTANCE IN CENTIMETERS)
401a	9	Preserved	30	—	—
401b	9	Preserved	44	—	—
402a	8	Preserved	47	—	—
402b	8	Preserved	55	—	—
402c	8	Preserved	70	+	11+
405a	9	Removed	72	+	12+
405b	9	Removed	81	+	8
405c	9	Removed	81	+	12 (80°)
401c	9	Preserved	102	+	10

The nerve tract innervating the transplanted muscle offered a distinctly grayish appearance when compared with the other spinal nerves. This is due to the presence of the degenerated motor fibers in the nerve. The difference in color is most striking between the two roots of the nerve, the gray of the degenerated, formerly ventral, root contrasting sharply with the bright white of the healthy dorsal root.

The threshold intensities required to obtain contractions in the transplanted muscles upon stimulating the nerves are given in table 1. Single shocks of threshold intensity were followed by conspicuous twitches of the muscles. Only a com-

paratively slight increase in the strength of the stimuli was necessary in order to obtain vigorous contractions.

The responses of the muscles were prompt and quick. No contracture or 'tonic' residue developed upon repeated stimulation. Generally speaking, there was nothing found in these contractions that would distinguish them essentially from the type usually obtained by stimulation of ordinary motor nerves. In two cases (402c, 405c) the contractions involved the whole muscle. In the other three cases the responses seemed to be limited to a smaller or larger part of the muscle next to the point of entrance of the nerve. This is easily explained by individual variations in the rate and completeness with which neuro-muscular connections are regenerated, since obviously those muscle fibers which surround the cut surface of the nerve, having the greatest chance to receive some of the new innervation, will be the first to show positive responses.

The muscular responses were obtained, no matter whether the stimuli were applied to the peripheral nerve or to the spinal ganglion. Stimulation applied to the dorsal roots was also successful in three cases; in the two cases where it failed to be effective, the dorsal roots probably have been damaged (stretched or constricted) in the dissection.

No differences in the responses was noticed between the cases in which the sympathetic system was intact and those in which it had been destroyed.

DISCUSSION

The results of the experiments demonstrate clearly that a purely sensory nerve is able to enter into such a connection with a muscle that excitations evoked in the nerve by electrical stimulation will produce true motor contractions in the muscle. There can be no doubt that the contractions observed in our experiments were due to nervous influences and not to an escape of the stimulating current. Sufficient evidence for this statement is contained in the following facts: The large distance separating the electrodes from the muscle, the

abolition of the responses by crushing the nerve, the fairly normal range of the threshold intensities, and the fact that no response whatsoever was obtained in the cases less than 55 days of age, even with the strongest stimuli. There can be no doubt either, that the contractions observed were due to excitations propagated along sensory fibers, all somatic motor fibers being destroyed as a result of the removal of the motor roots. The dislocation of the roots from within the vertebral canal into the body cavity precluded the possibility of their being refilled by regenerating motor fibers. In three of the functional cases the sympathetic fibers formerly contained in the nerve trunk have also been eliminated by the destruction of the sympathetic ganglia and rami. In these cases it is, therefore, evident that the nerves innervating the transplanted muscles were purely somatic sensory in the strictest sense. But even in the cases where the sympathetic fibers had been preserved it is obvious that the muscular responses were mediated by the somatic sensory fibers, since in these specimens, the contractions could be elicited on the isolated preparation by stimulating the dorsal root, which is far proximal to the union of the peripheral sympathetic fibers with the nerve.

The results corroborate the histological findings of Boeke ('17), who had demonstrated that connections between sensory nerve fibers and muscle fibers are possible, and supplement Boeke's findings by showing that across those connections nervous excitations can actually be transmitted, yielding normal motor effects. To judge from these results, it is very likely that the contractions of the tongue muscles obtained by Philipeaux and Vulpian (1863) and by Rosenthal (1864) upon stimulation of the lingual nerve regenerated into the hypoglossic were also true motor contractions, effected by way of the sensory fibers, rather than 'pseudomotor' contractions, effected by chorda fibers; the more so, as those contractions have been reported as prompt and quick, as contrasted with the slow and sluggish (van Rijnberk, '15) 'pseudomotor' reactions, a difference the significance of which has been emphasized already by Rogowicz (1885).

The fact now definitely established that sensory fibers can effectively innervate muscles again stresses the view that there is no exclusive selectivity, acceptance or rejection, involved as a controlling principle in the formation of regenerative connections between nerve fibers and peripheral organs. At least, under conditions where only a single type of nerve fiber is available, as in the above experiments, no rejection of 'heterogeneous' fibers was manifested by the peripheral organ. It has been claimed, however, that when both types of fibers, sensory as well as motor, are present, they will become selectively distributed to their respective peripheries (Bethe, '07). Whether and to what extent this holds, it would require more extensive experiments to decide. That selective distribution as a mere fact after all exists, is strikingly illustrated by nerve ontogenesis as a result of which motor nerves lead to motor organs and sensory nerves to sensory organs. But how this condition is achieved is still entirely obscure. It is unfounded speculation to assume that particular types of nerve fibers are picked out selectively by their respective peripheral fields from a common pool. No trace of such predilection was noticed in tissue culture (P. Weiss, '34), and experiences with regenerating nerves as reported in this paper are certainly not in favor of any such view, either. We probably shall not reach a clear understanding of these matters until many more basic facts have been gathered, and it is deplorable that more work is not devoted to these fundamental problems.

Another question, of course, is whether heterogeneous connections are established as readily and as easily as homogeneous connections. In the experiments reported above the minimum period within which sensory fibers effected functional connections with muscle fibers amounted to about 2 months. In my earlier work where motor nerves had been inserted into muscles under conditions similar to the present experiments (P. Weiss, '31), only about 1 month was required until the muscle became excitable from the nerve. This difference seems to indicate that in order to achieve a motor

connection with a muscle fiber more time is required for sensory fibers than for motor fibers. However, sensory nerve fibers seem to regenerate at a lower rate than motor fibers, and in addition the animals used for the recent experiments with sensory nerves were somewhat larger and suffered from nematode infections, factors which are likely to retard the processes of regeneration and recovery. Boeke mentions that in his experiments the outgrowth of sensory fibers into a motor nerve apparently did not take place as smoothly as in homogeneous nerve regeneration ('17, p. 53). But we must remember that in Boeke's cases, in contradistinction to ours, the sensory fibers had been united with remainders of motor nerve fibers and not with muscle fibers directly, so that the two sets are not strictly comparable. At any rate, from our own experiments it cannot yet be safely concluded that heterogeneous nerve connections meet with greater difficulties than homogeneous ones.

While my earlier experiments, dealing with the innervation of muscles by dorsal roots (P. Weiss, '35), had already proved the fact that transmission of excitations from a sensory nerve fiber to a muscle is possible in the centripetal direction, which is the normal direction of flow of excitations in these nerves, the present results bring the additional information that transmission in the opposite direction, i.e., toward the periphery, is likewise possible. We must conclude that at the peripheral end of a sensory neurone transmission of excitations can take place in different directions, depending on the sort of end organ with which the fiber is connected: centripetally, from the end organ toward the fiber, if that organ is a sensory one, and centrifugally, away from the fiber, if the organ is a motor one, as in our present experiments. From this it becomes evident that if irreciprocity of transmission in a chain of neurones and end organs is due to one-way mechanisms in the synapses, such mechanisms can certainly not be intrinsic properties of the neurones themselves, but must be assumed to be determined, for each branch of the neurone separately, by the character of the structures with which the branches

connect. It is logical to expect that if in a combined experiment a spinal ganglion were isolated and the stump of the dorsal root inserted into one muscle and the stump of the peripheral sensory nerve inserted into another muscle, excitations of this nerve would be transmitted in both directions to both muscles.

SUMMARY

Mixed nerves were turned into purely sensory nerves by destroying the motor roots (and, in a number of cases, the sympathetic ganglia and connections also). These nerves were then severed peripherally and the cut surface was inserted into a denervated (transplanted) muscle. The inserted sensory fibers formed motor connections with the muscle. From 10 weeks on after the operation these connections were found to be functional: Electrical stimulation (single induction shocks) applied to the nerve yielded normal motor contraction in the muscle. It has been definitely proved, therefore, that sensory nerve fibers can transmit motor effects to muscles.

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STUDIES ON CELL STRUCTURE BY THE FREEZING-DRYING METHOD

VI. THE PREPARATION AND PROPERTIES OF MITOCHONDRIA

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As indicated in the preceding paper, the mitochondria of the liver of the guinea pig and rabbit are wholly insoluble in a 0.85 solution of sodium chloride. In order to obtain mitochondria for examination, it is only necessary to extract by differential centrifugation the emulsion of guinea pig liver described in the previous article.

The emulsion is prepared by grinding or crushing in a mortar fresh liver which has been washed free of blood by perfusion, or by kneading through bolting silk. This emulsion, as stated in a previous paper, contains fragments of small aggregations of liver cells, individual liver cells in great abundance, large numbers of nuclei released from cytoplasm, a few blood corpuscles and much reticulum and mitochondria. The salt solution used for this preparation has a pH of about 6, so has a slightly acid reaction. Distinctly alkaline Ringer solutions are quite unsuited for the purpose and salt solutions made with conductivity water are not so successful as solutions made with ordinary distilled water, having the reaction indicated above.

Centrifugation of this emulsion at low speed for a period of 3 minutes sediments the majority of cell masses. If an attempt be made to get out the suspended cells at one operation, a great loss of mitochondria ensues, so it is better to rid the suspension of the groups of intact cells, individual

[1] This research was aided by a grant to The University of Chicago by the Rockefeller Foundation.

cell nuclei, blood corpuscles and connective tissue by repeated short centrifugations at moderate speeds. As indicated, a preliminary centrifugation at low speed throws down the majority of the cell masses. A second centrifugation for about 10 minutes, or a series of 3-minute centrifugations at a slightly higher speed throws down practically all of the corpuscles, and all of the intact cells and free nuclei, leaving a suspension which contains, in addition to its content of soluble proteins and glycogen, mitochondria with a few fragments of connective tissue and some fat. A considerable loss of available mitochondria is incurred in getting rid of cell masses and cell nuclei. As will be seen, the contamination of the final product with these elements is easily discerned by an examination of its fat content.

Having got rid of the gross cell debris, the final supernatant fluid containing the mitochondria in suspension is centrifuged at high speed for a longer period. By this means is obtained a precipitate consisting of mitochondria, some entangled fat, some fragments of cells, and a small quantity of connective tissue.

Preparations of the liver of the pig made in the same way show a large content of finely divided reticulum mixed with mitochondria, but in the liver of the guinea pig there is very little reticulum carried into this final precipitate.

The supernatant fluid is poured off and the precipitate of mitochondria repeatedly washed by suspension in 0.85 per cent salt solution and centrifugation. This washing is continued until the wash water no longer shows traces of soluble protein substances. The mitochondria are finally washed with distilled water slightly acidified with acetic acid, and then dried rapidly in vacuo over phosphorus pentoxide in the centrifuge tubes. The mass dries to a cake which can be separated and easily shaken out of the tube.

In carrying out this process it is necessary to use only salt solutions which have been thoroughly cooled and to proceed to the end product with as much speed as possible.

The preparation can be tested at any moment at any part of the process by spreading a portion of it on a slide, fixing with osmic acid and staining with anilin-acid-fuchsin and methyl green. This will show whether we have a pure suspension of mitochondria or whether the suspension is contaminated by the presence of nuclei, larger cell fragments or blood corpuscles. As a rule, it is not difficult to get rid of these contaminations and to obtain a pure precipitate of mitochondria. The preparation when dried in vacuo can also be broken up and separated into its constituents in salt solution and stained on the slide for identification.

During this process the mitochondria swell slightly, change from rods to ovals or cone-shaped structures, but do not fuse with one another. One gains the impression that there is a little loss of material from them since the staining with acid fuchsin, though intense, is less so than the staining of mitochondria from fresh smear of crushed cells. On the contrary, this difference in staining power may simply be due to the swelling of mitochondria which takes place in the salt solution which is slightly hypotonic. A disadvantage of the purely mechanical separation of this material is to be found in the fact that in the frequent changes of salt solution and resuspensions it is impossible to completely exclude the entrance of foreign material into the emulsions.

In view of the fact that the majority of cytologists regard these bodies as being largely composed of lecithin-like materials, and that Bensley and Gersh in a previous investigation found evidence of a large content of protein and no evidence of lipoid content, it has been of much interest to determine the total fat content and its nature. For this purpose weighed quantities of mitochondria were extracted successively for long periods in a Soxhlet apparatus with hot alcohol, ether and chloroform. The extracts were then combined, evaporated to dryness in a weighed evaporating dish, and the total weight determined. The results of three such determinations were as follows:

Preparation 1: 0.0777 gm. of mitochondria yielded 0.0322 gm. of mitochondria, or 42.7 per cent of the dried weight of the mitochondria as fatty substances.

Preparation 2: 0.1162 gm. of mitochondria yielded 0.0514 gm. of extractive, equivalent to 44.2 per cent of the total weight of the mitochondria.

Preparation 3: 0.0665 gm. of mitochondria yielded 0.0292 gm. of extractive, equivalent to 43.9 per cent of the total weight of the mitochondria.

So we may say with some confidence that the mitochondria of the liver of the guinea pig contain an average of 43.6 per cent of their dried weight of substances soluble in alcohol, ether and chloroform.

It should be pointed out that the majority of this material was extracted in the first extract of hot alcohol, as stated by Bloor and Theis. But in one instance 22.5 per cent of the total fats were recovered from the ether and choroform extracts; in the other instances the alcohol extraction was more prolonged and the ether and choroform extractions yielded little additional material.

The first preparation of mitochondrial fat consisting of 32.2 mg. was dissolved in 20 cc. of chloroform and acetone added in excess. To our great surprise, the copious precipitate of lecithin expected did not occur. The solution became slightly opalescent and after standing for a period of 15 hours in the refrigerator deposited a small amount of white precipitate. This was recovered by centrifugation and washed with acetone, redissolved in chloroform and evaporated to dryness in a previously weighed evaporating dish and 2.8 mg. of material were recovered. The small yield of this material aroused doubts as to its being the expected lecithin and cephalin, and this was particularly emphasized by the fact that two other preparations of mitochondria fat treated in the same way yielded no precipitate with acetone whatever. Accordingly, we requested Dr. Preston Kyes to apply the test for lecithin of activation of cobra venom hemolysin to these preparations. For this purpose he used in one instance

10 mg. of mitochondria. This product, shaken for 3 hours in 10-cc. salt solution, and tested for activation of cobra venom hemolysin for sheep corpuscles, showed no hemolysis. Ten mg. of the same material extracted with chloroform and with hot methyl alcohol showed no precipitate with acetone and the extractive suspended in salt solution showed a moderate amount of activation. He was in doubt whether this was lecithin activation or indirect fatty acid activation, or due to a trace of methyl alcohol.

The small amount of acetone-insoluble components obtained from a total of 32.2 mg. of mitochondrial fat showed no activation, and was therefore not lecithin or cephalin.

Another specimen containing 0.0698 gm. of mitochondrial fat showed a moderate hemolytic capacity by itself and an increased hemolytic capacity in combination with cobra venom which Doctor Kyes estimated to be about equivalent to the secondary activation due to free fatty acid.

The result of these experiments and the additional fact that a preparation of mitchondria in which the red blood corpuscles had been incompletely removed gave a copious precipitate with acetone, indicates that the mitochondria of the guinea pig liver do not contain either lecithin or cephalin. The distribution of substances in the alcohol-ether-chloroform extracts has not yet been determined, but is under way. Larger quantities than we have prepared up to the present are necessary for this investigation.

Osmic acid is reduced by dried mitochondria to a brown color and the separated fat from mitochondria also reduces osmic acid, so that the browning of mitochondria in preparations fixed in solutions containing osmic acid is due in part at least to the direct reduction of osmic acid by an unsaturated fatty compound in the mitochondria.

Freshly prepared mitochondria of the guinea pig liver are immediately soluble in N/2000 ammonia, and the constituent proteins can be separated by this means from the small contamination of foreign material and of reticulum which is inevitable in preparations by purely mechanical

methods. In order to accomplish this separation, however, it is necessary to proceed at once with it within a period of 24 hours after the whole operation is begun, because the mitochondria rapidly, in the moist condition, even when kept at low temperatures, pass into a state in which they are insoluble even in alkalis. In this state they can only be put in solution by a treatment which results in profound modification of the constituent compounds. It is better in preparing the constituent proteins of mitochondria to finish the operation in 1 day; that is, the separation of the mitochondria, their solution, and the reprecipitation of their constituent proteins.

To prepare the specific proteins of mitochondria, the following method was found effective: After the final washing of the mitochondrial suspension, the mitochondria are collected by centrifugation, are suspended in a known quantity of distilled water and enough standard ammonia solution added to this known volume to bring the content of ammonia up to a N/2000. The mitochondria dissolve almost instantly, and the residue of mechanical contamination in the original preparation may be separated immediately by centrifugation. The mitochondria form a slightly opalescent solution. Some of the constituent fat is saponified in this process, and lost in subsequent washings, but a great deal is carried over into the final preparation of the mitochondrial proteins.

The solution cleared by centrifugation is carefully neutralized. When the pH 6.6, as determined by the use of brom cresyl purple as an indicator and comparison with standard buffer solutions is reached, a precipitate makes its appearance. If this precipitate be now separated by centrifugation and additional acid be added, a second precipitate is obtained. The exact pH at which this second precipitate appears has not been determined, but it is sufficient to indicate at the present time that there are two protein substances in mitochondria having different isolelectric points which can be separated be careful neutralization. These proteins are readily soluble in excess of acid and, like the mitochondria from which they are obtained, pass rapidly, even at low tempera-

tures, into an insoluble form. They can only be recovered if the operation is effected with the utmost speed and if the materials are kept constantly at low temperatures.

The chemical investigation of these proteins of mitochondria is also proceeding and will be reported on later.

SUMMARY

Mitochondria in pure form have been separated from the other cell constituents of the liver cell of the guinea pig and examined chemically. They contain no lecithin and cephalin, but have on an average 43.6 per cent of fatty substances soluble in hot alcohol, ether and chloroform. They also contain two proteins which may be separated from one another by differences in their isoelectric points. These proteins and the original mitochondria are soluble in very dilute alkaline solutions and pass readily in the moist conditions into an insoluble state, even at low temperatures.

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THE EFFECT OF THEELIN ON THE MAMMARY RUDIMENTS OF MALE MICE DIFFERING IN SUSCEPTIBILITY TO TUMOR DEVELOPMENT

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THREE PLATES (TWENTY-TWO FIGURES)

The female sex hormone, theelin, has been found to induce growth changes in the mammary glands, uterus and vagina of ovariectomized animals, closely duplicating those conditions normally observed during certain stages of the sexual cycle. Theelin is a specific growth stimulant for these tissues (Allen, '32). It seems possible that theelin might stimulate excessive growth or abnormal growth when present for long periods.

It is frequently considered that the tendency to develop malignant growth is determined genetically. The incidence of such growth is very high in the mammary glands of female mice of some carefully inbred strains and extremely low in other strains. The incidence of mammary tumors in all male mice is also extremely low, perhaps due to the very rudimentary condition of their mammary glands. Thus, in studying the effect of long continued theelin injections upon the mammary glands, three strains of male mice differing in the inherent tendency for tumor development were selected in an attempt to simultaneously study the possibility that theelin might induce atypical growth.

The effect of estrogenic preparations upon the mammary glands has been observed by a number of investigators. For

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a review of these investigations, reference is made to Turner ('32). Reference here will be made only to the work applying directly to the effect of theelin on the mammary glands of the mouse.

The first evidence that theelin had some growth stimulating effect upon the mammary glands of the mouse was presented by Allen et al. ('24). Turner et al. ('32) reported the influence of two estrogenic preparations (theelin and theelol) on the mammary glands of male mice. Three male mice were injected with 5 rat units of theelin daily. One animal was killed after 15 and one after 21 days of treatment. The third animal was then given 10 rat units of theelin daily for 41 days. The mammary glands of the first two mice showed no growth effects, while the mouse receiving the larger dose possessed an extensively developed duct system. It seemed probable from this preliminary experiment that a large amount of theelin might be necessary to induce the growth of the mammary glands of the male mouse. Theelol stimulated a similar growth of the mammary glands.

The growth of the galactaphores was observed in castrated male and female mice by Bradbury ('32). Though he made no statement concerning the length of treatment, he observed that the proliferation induced was confined entirely to the ducts.

Lacassange ('32) injected three male and two female mice of a cancer susceptible strain with 1/20 cc. of an oil solution of folliculine benzoate which contained 0.6 mg. of the crystalline product per cubic centimeter. Injections were made at weekly intervals. After the injections had been continued for 5 and 6 months, tumors diagnosed as adeno-carcinomata developed in the three males. Lacassange made no definite statements relating to the development of the glands induced by this treatment.

Turner and Gomez ('34) observed that even extended injections of theelin in large quantities induced only growth of the duct system of the glands of male mice. The hormone of the corpus luteum was required in addition to stimulate

the development of the smaller lobular ducts and alveoli. They reported that comparatively large amounts of theelin were necessary, 10 rat units or over per day, to induce growth of the galactaphores, and assumed that the mammary glands of the male mouse, which remained extremely rudimentary throughout life, required a relatively high hormone threshold.

The effect of an estrogenic preparation upon the mammary glands of post-pubertal female mice was made by Goormaghtigh and Amerlinck ('30). Daily injections of 6 rat units of the preparation induced definite adenomatous growths after a period of 34 days or more. They were inclined to interpret their results as due to the effects of the corpora lutea induced to persist by such treatment.

In the present experiment two points were investigated: first, what influence will long continued injections of theelin have upon the extent of development of mammary glands of the male mouse; and, second, will theelin induce in addition to the normal growth of the glands, atypical growths in genetically susceptible animals?

PROCEDURE

Mice of three different strains were selected. Two of the strains (C_3H and A) gave high incidence of spontaneous mammary cancer in the females. In the third strain (CBA) a mammary tumor has never been observed in the females. In all three strains spontaneous cancers do not develop normally in the male. The albino mice strain (A) had been inbred for forty-three generations. The mice of the other cancer susceptible strain (C_3H) had been inbred for twenty-eight to thirty generations. The mice used from the cancer resistant strain (CBA) had been inbred for from twenty-seven to twenty-nine generations, inclusive. Sixteen young males (53 to 67 days old) were selected from each strain. Two untreated mice of each strain were killed shortly after beginning the experiment and their mammary glands studied as controls. At the end of the experiment one untreated mouse of each strain was killed as a control.

The remaining thirteen mice of each strain received theelin [3] injections for from 15 to 147 days. All injections were made subcutaneously twice daily. Since very small quantities of theelin were used, one part was diluted with four parts of physiological saline to enable greater accuracy of injection. Nine mice of each strain received 2 rat units of theelin daily and four received 1, 3, 4 and 5 rat units, respectively, each day. Animals were killed on the 15th, 20th, 27th, 36th, 61st and 75th days of the injection period. The remaining animals were obtained shortly before or after death (table 1).

The mammary glands were studied largely as whole gland preparations. The entire skins were fixed in Bouin's fluid and the glands were dissected from the fixed skins, stained in Mayer's hemalum and mounted in balsam after destaining and clearing. Such preparations may be studied under considerable magnification.

The following tissues were also removed at autopsy and fixed in Bouin's fluid: seminal vesicles, Cowper's glands, testes, epididymis, prostate, thyroid gland and the left suprarenal. The tissues were cut at 10 μ and stained with haematoxylin and eosin.

RESULTS

The mammary glands of the male mouse consist of small rudimentary ducts which may be straight or branched. These ducts are free in the subcutaneous connective tissue as they lose their cutaneous attachment in embryonic development (Turner and Gomez, '33). Nipples never develop. Though the mammary glands of males undergo considerable growth in some species, in the mouse they remain rudimentary throughout life. The mammary glands of untreated male mice under 100 days of age, over 200 days, and over 700 days, are shown in figures 1 to 6. Mammary glands removed from male mice from the twelfth to fifteenth days of postnatal life are equally well developed.

[3] We wish to express our appreciation to Parke-Davis Company and to Dr. Oliver Kamm for the supplies of theelin used in this experiment.

TABLE 1

Results

MOUSE NUMBER	AGE AT START (DAYS)	AGE AT DEATH (DAYS)	PERIOD OF TREATMENT (DAYS)	RAT UNITS DAILY	TOTAL THEELIN IN RAT UNITS
		A			
100959	59	137	75	1	75
100829	58	198 died	141	2	282
100795	65	101	36	3	108
101043	61	Died and not recovered			
101042	61				
100960	59	198	198	2	278
100827	58	193 died	134	2	268
100796	64	204 died	140	2	280
101044	61	88	27	2	54
101045	61	200 died	139	2	278
100961	59	179	120	2	240
100797	65	126	61	2	122
101046	61	77	15	2	30
101174		70 controls			
100973		92 controls			
100946		205 controls			
		C_3H			
100850	67	202 died	135	1	135
100977	56	195 died	139	2	278
100975	56	204 died	148	3	444
100974	56	189	133	4	520.5
100810	61	80	20	5	100
100854	56	194 died	138	2	276
101108	64	90	27	2	54
101107	64	200 died	136	2	272
101214	53	199	146	2	292
100979	56	195 died	139	2	278
100811	61	200	139	2	278
101215	53	114	61	2	122
100978	56	200	144	2	288
101355		82 controls			
100357		211 controls			
100104		60 controls			
		CBA			
100786	56	191 died	135	1	135
101218	55	70	15	2	30
101221	55	194	139	3	417
95931	67	103	36	4	144
95933	67	103	36	5	180
100790	56	197	141	2	282
101220	55	190	141	2	282
101222	55	202	147	2	294
95932	67	215	148	2	296
101223	55	Died and not recovered			
100817	61	200 died	139	2	278
100909	59	Died and not recovered			
101815		76 controls			
100908		85 controls			
101178		200 controls			

The female mice of the three strains studied normally have ten mammary glands and ten nipples. In the A strain and to a lesser extent in the C_3H strain, one or both of the third pair of glands, however, tend to remain more or less rudimentary. As the glands of the females have been followed closely only through the earlier growth stages, it is impossible to say whether or not this is due to a delayed growth. Occasionally a female of the A strain will have a deficiency of one or both of this same pair of glands. Similarly, it was not possible to find all of the expected mammary rudiments in the male. The males of all strains were similar in this respect. Likewise, the third pair of glands was most frequently deficient. As these rudiments were very small, it is possible that they might have been overlooked, even though dissections of the stained glands were made under a binocular dissecting microscope.

Two animals (A and CBA strains) were killed after being injected for 15 days. Each animal had received a total of 30 rat units of theelin. The mammary glands had developed rapidly. Distinct terminal buds capped the growing ducts (figs. 7 and 8). In the mouse from the A strain the third pair of glands still were very small, being no larger than the mammary glands of the control animals. One of the third pair of glands of the CBA was likewise small. The other could not be located.

A mouse from the C_3H strain was killed after receiving 100 rat units of theelin in 20 days. The mammary glands resembled those removed at 15 days, though they were slightly larger (fig. 9). The third pair of glands was not found.

Two mice, one from the C_3H and one from the A strain, were killed after receiving 54 rat units of theelin in 27 days. The glands were larger than at the 20-day stage. The ends of the ducts were still bulbous and appeared to be rapidly proliferating (fig. 10). In the mouse of the A strain the third pair of glands was slightly smaller than the other glands.

On the thirty-sixth day of injections the mammary glands were slightly larger. One of the mice (from the A strain)

had received 3 rat units of theelin daily, one (CBA) 4 rat units, and one (CBA) 5 rat units. The extent of the glands was quite uniform, though the dosages varied. An increased number of small lateral buds had developed from the larger ducts (figs. 11 and 12).

After 61 days of treatment, the mammary glands had further increased in size. An A strain and a C_3H strain animal were killed at this time. Though both animals had received 2 rat units of theelin daily and the gland areas were approximately uniform in size, many small lateral sprouts had developed in the A strain mouse (fig. 15). The third pair of glands could not be located in the C_3H mouse, and only one of the pair was found in the albino. This one gland was about one-fourth the size of the other mammary glands.

An A strain mouse receiving but 1 rat unit daily was killed on the seventy-fifth day of treatment. The gland area was similar to that of the mouse treated for 61 days (fig. 16). A few enlarged terminal buds were present, indicating active proliferation.

The remaining mice were killed or died from the 120th to 147th days of injection. Since for some unknown reason they became sickly at this time, most of them were killed between the 135th to 147th days of treatment. In all cases, however, the duct system of the glands was well developed (figs. 13, 17 and 18). Frequently one or both of the third pair of mammary glands could not be located. Very few enlarged end buds were observed, which led to the assumption that growth had practically ceased. There was considerable variation in the number of lateral sprouts appearing from the larger ducts, but in no instance were they more numerous than in the mice injected for 61 days.

In three mice (C_3H) localized atypical appearing areas were observed (figs. 17 and 18). Grossly, these areas resembled small lobules of mammary tissue. In two of the mice they were located on the ducts (figs. 21 and 22). In the third mouse they were terminal enlargements (figs. 19 and 20). Upon sectioning and microscopic examination, these

atypical enlargements markedly resembled small, secreting lobules (figs. 19A to 22A). Definite alveoli had formed. The aveolar epithelium contained numerous fat globules and the lumina were filled with secretion (figs. 21A and 22A). No mitotic figures could be definitely observed. These 'atypical' structures were observed after 139 to 146 days of treatment. As they were all very small, they could not be observed until the glands had been removed and fixed.

No deviations from the normal, which might be attributed to theelin treatment, were found in any of the other tissues studied except the testes. A critical study of the tissues removed from the animals obtained some time after death, however, could not be made. Animals injected for 36 days or more showed degenerative changes in a number of the seminiferous tubules. The tubules first affected were the most peripheral. That the testes were not severely damaged was shown by the presence of normal spermatids in the lumina of the tubules in all animals.

DISCUSSION

The mammary rudiments of the male mouse are very small at all times during the life of the animal. They do not develop after the mouse is 100 days old, and very little, if at all, after the fifteenth day. Further, no marked regression has been observed to occur in the rudiments following this time. In this manner the mouse differs considerably from other species of experimental animals in which the course of mammary development has been closely observed. In the rat, Turner and Schultz ('31) have described a considerable development of the mammary glands of the male. An increase in the size of the mammary glands of male rabbits with increasing age and weight has been observed by Turner and Frank ('30). Several investigators have observed the extensive development of the mammary glands of male guinea pigs.

That theelin is necessary for the stimulation of all mammary gland growth after their embryonic differentiation cannot be stated at the present time. Even in the complete

absence of a known ovarian hormonal stimulation, the mammary glands of some species may develop to an extent proportional to the rest of the body. In the mouse, this is apparently not the case. That the development of the mammary glands of normal, untreated, male rats may be stimulated by theelin has been suggested by Schultz and Turner ('33), who obtained theelin in the excreta of such animals.

Theelin will, however, invoke a marked development of the male mammary rudiments. The concentration or amount necessary cannot be determined from the present investigation, as all injections were equal to or above the threshold level. There was but little evidence that the mammary glands of the mice receiving 4 and 5 rat units daily developed more rapidly than those receiving 1 and 2 rat units. There is more evidence that the type of development may be affected. Thus, the mice injected with larger amounts of theelin for 36 days showed glands with very numerous, small, lateral branches, whereas the mice injected with smaller amounts showed glands with long, smooth ducts. There were considerable variations in degree of development, however.

The large amounts of theelin required to stimulate growth of the mammary glands of the male mice used by Turner and Gomez ('34) cannot be explained. It appears doubtful that it may be due to difference in assay. Their hormone was dissolved in oil and assayed in rats. The theelin used in the present investigation was assayed by Parke-Davis and standardized at 50 rat units per cubic centimeter. Upon assay in ovariectomized mice it was found to contain more than 150 mouse units per cubic centimeter, or 3 mouse units per rat unit of the original assay of the manufacturer.

The mammary glands of individuals even in the same strain varied somewhat in the number of small lateral buds developing from the larger galactaphores. The development of these buds was apparently not observed by either Turner and Gomez or Bradbury. The three strains used showed no variation in the number of small duct-buds appearing. Even in the C_3H strain, where the localized atypical enlargements ap-

peared, the small buds were frequently lacking (figs. 13, 17 and 18).

The mammary glands appeared to reach their maximum extent after being stimulated for a period of 60 to 75 days. Following this time the mammary glands persisted with but slight change. The large end buds of the rapidly growing glands had entirely disappeared. The ducts appeared to be undergoing no further proliferation. In a few instances an increased number of small branch ducts appeared, but this was not uniform. In other individuals slight retrogressive changes appeared. In the rabbit a regression of the mammary glands after they have been developed by theelin injections has been observed, even though the treatment was continued (Turner and Frank, '32).

As the expected number of mammary glands has not been found in most of the male mice, even after extended stimulation, it was assumed that they had been lost during embryonic development or failed to develop at that time. The deficiencies, however, were much more frequent than in the females of the same strains. In only one of the strains (A) has less than the usual number of glands been observed in females, and then in but two instances. In the males, as in the females, the glands of the third pair were most frequently lacking. In two of the strains (A and C_3H) the third pair of glands are frequently much smaller in size when present. This same condition has also been observed in the males. The rudiments of the third pair of glands, when present, appeared to be less susceptible to stimulation.

The localized nodules were composed of groups of normal appearing and arranged acini. They were atypical in that they were localized excessive developments. Grossly, they resembled lobules similar to those appearing late in pregnancy. Theelin is thus capable of stimulating certain localized areas of the glands of this one strain of mice, at least, to a much greater extent than other areas. In one mouse one area was found on each of two glands. Five or more areas were observed at the distal ends of the ducts of two glands of a second

animal, and but one area was observed in a third animal. That certain cells of the glands may be more sensitive to stimulation than others was indicated by the random location of the growths.

From the above observations it cannot be stated that theelin will develop the mammary glands to a point where tumors will be produced or that theelin will stimulate the development of mammary tumors. Previous investigations have shown that theelin will stimulate the growth of the duct system of the mammary glands. We have found that it will also stimulate in one strain of mice, at least, the formation of functional alveoli in certain localized areas.

SUMMARY

1. The observation that theelin will stimulate the mammary rudiments of the male mouse has been verified in three strains of male mice. No strain variation was observed in the rate or extent of development induced in the mammary ducts. The mammary glands of the theelin treated males undergo a gradual growth for 60 to 75 days. After this time they show little change.

2. The amount of theelin required to stimulate the growth of the mammary glands of the strains used is much less than that reported by others, 1 rat unit or 3 mouse units daily producing growth.

3. In the normal male mouse the mammary rudiments have been observed to remain unchanged from the 13th to the 735th days. All of the mammary glands are apparently not developed in the male, as even after theelin injection some of them cannot be found. This deficiency most frequently involves the third pair of glands.

4. Atypical growths, somewhat resembling normal and functional lobules, appeared on localized areas of the mammary duct system of the three male mice of one of the strains injected with theelin.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

The following drawings are silhouette reproductions of projected mammary gland preparations. Enlarged 5.8 times.

1 to 6 Mammary glands of six normal control male mice of the following strains and ages: 1, CBA (101815), 76 days of age; 2, CBA (100908), 85 days of age; 3, CBA (100178), 200 days of age; 4, C_3H (100357), 211 days of age; 5, $C_{12}G$ (79629), 735 days of age; 6, five glands of an A strain mouse (100946) at 205 days of age.

7 One mammary gland of each of the second (A) and third (B) pair from a male mouse of the CBA strain (100218) after the injection of 30 rat units of theelin during 15 days. The enlarged end buds are characteristic of rapidly proliferating glands. The mammary glands of the third pair are still very small, apparently not reacting to the injected theelin.

8 Same as figure 7 above for a male mouse of the A strain (101046).

9 A mammary gland of the second pair from a male mouse of the C_3H strain, which had received 100 rat units of theelin in 20 days.

10 One of the mammary glands of the second pair from a male mouse of the A strain (101044). This mouse had received 54 rat units of theelin in 27 days.

11 A mammary gland from the second pair from a male mouse of the CBA strain (95931). This mouse had received 144 rat units of theelin in 36 days, or 4 rat units daily.

12 A mammary gland from the second pair of a male mouse of the CBA strain (95933). This mouse had received 180 rat units of theelin in 36 days.

1
2
3
4
5
6
B
11
A
7
9
10
B
12
A
8

PLATE 2

EXPLANATION OF FIGURES

13 One of the mammary glands of the second pair from a male mouse of the C_3H strain (101214), which had received 292 rat units of theelin in 146 days. Six areas of 'atypical' growth were observed in the third pair of glands of this individual. Two of these growths are shown in figures 19 and 20.

14 A mammary gland from the second (A) and third (B) pair of a normal 31-day-old female mouse. Note the presence of the nipple on the female mouse and the relative sizes of the glands.

15 A mammary gland from a male mouse of the A strain (100797) after the mouse had received 122 rat units of theelin in 61 days.

16 A mammary gland from a male mouse of the A strain (100959) which had received 75 rat units of theelin in 75 days, or 1 rat unit daily.

13
15
A
14
B
16

PLATE 3

EXPLANATION OF FIGURES

17 A mammary gland from a male mouse of the C_3H strain (100811) after 278 rat units of theelin had been injected during 139 days. Comparatively few lateral sprouts were observed along the ducts. The enlargement is the location of one of the 'atypical' growths.

18 One of the mammary glands of the first pair from the same mouse described in figure 17. The 'atypical' area is shown (A). Also see figures 22 and 22A.

19, 19A, 20, 20A Two of the six 'atypical' growths observed in mouse (101214). All of these excessive growths were terminally located. Drawings enlarged 21 times. Photomicrographs × 92.

21 and 21A A condition similar to that described for figure 22. This growth was observed in the mammary gland shown in figure 17. The alveoli with vacuolated epithelial cells surround the large, deeply stained, central duct. Drawing × 21. Photomicrograph × 92.

22 and 22A A drawing of the nodular growth and a photomicrograph from a section of the growth observed in the mouse described with figure 18. The area somewhat resembled a normal, functional lobule of alveoli. The alveoli were partially filled with secretion. Drawing × 21. Photomicrograph × 92.

17

18

20A

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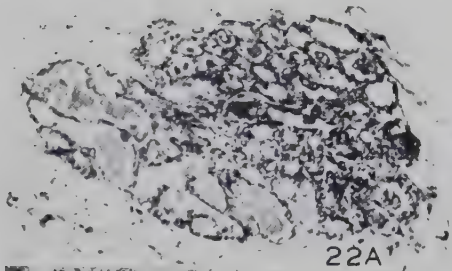
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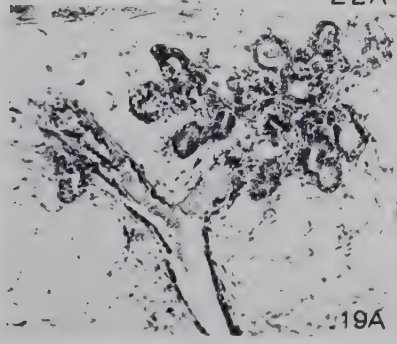
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INTERCALATED DISCS OF THE HEART MUSCLE OF THE GUINEA PIG. COUNTS OF THE NUMBER OF DISCS PRESENT AT EIGHT AGES OF NORMAL ANIMALS AND IN TWO EXPERIMENTAL GROUPS [1]

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ONE FIGURE

INTRODUCTION

Notwithstanding the great amount of careful study of intercalated discs of heart muscle, their significance is far from being agreed upon at the present time. There is general agreement (Aschoff, '06; Cohn, '09; Jordan, '11; Jordan and Banks, '17); and Witte, '19) that the number of discs increases with the age of the animal, but as far as we know statements in regard to this point are wholly general in nature, no actual counts of discs from hearts of animals of known ages having been reported. Therefore, it seemed worth while to establish a record of the number of discs appearing in heart muscle of normal animals of known ages, as only with such information available would experimental work as suggested by the statement of Marcus ('25), "Nur bei Steigerung oder Störung der Funktion kommt es dann zu den typischen Glanzstreifen . . . ." become significant.

In the present work counts have been made of the number of intercalated discs present in a given area of ventricular muscle in forty-eight normal guinea pigs of eight different ages. In addition, an attempt has been made to bring about a variation from the number normally occurring in the heart by experimentally disturbing the activity of the heart in two groups of six animals each.

[1] Contributions from the Department of Zoölogy, Smith College, No. 183.

MATERIAL AND METHODS

Ventricular muscle was taken from the heart of the guinea pig immediately upon the death of the animal which was killed by a blow on the head. Ventricular muscle was studied from animals of the following ages: late embryonic, newborn, 1 week, 2 weeks, 3 weeks, 4 weeks, 8 weeks and 12 weeks. Six animals of each age were used. Twelve experimental animals, following experimentation of 2 weeks' duration, were 4 weeks old when killed.

Zimmermann's technique as described by Palczewska ('10) proved most successful, and was employed on all the material. Sections were cut 7 to 10 μ in thickness. The nuclei and intercalated discs stained a deep purple, the striations a lighter purple, and the sarcoplasm a very pale lavender.

Five slides were made of ventricular muscle of each of the forty-eight normal guinea pigs and the twelve experimental guinea pigs. A Spencer 1.8 mm. objective and the compensatory ocular × 10 were used. A Leitz ocular micrometer was used to limit the field to an area of 0.0085 sq.mm. Twenty-five fields were taken at random from the five slides. In general, only one or possibly two fields were taken from a single section.

Counts of the number of intercalated discs occurring at one optical level in an area of 0.0085 sq.mm. were made. One hundred such areas were counted for one animal at each age. The mean and standard deviation of the number of intercalated discs occurring in 100 areas and in twenty-five areas were calculated. Since the difference was not of statistical significance, twenty-five areas were counted in the remaining animals.

In addition to these studies on the hearts from forty-eight normal animals, we endeavored, by experimentally disturbing the normal activity of the heart, to bring about a variation from the number of intercalated discs occurring in the normal heart. Six animals were injected intraperitoneally with 1 cc. of a 0.01 per cent thyroxin solution per 100 gm. of body weight and six animals were given electric shocks for 1 hour daily by means of a tetanizing current.

STATISTICAL DATA

Normal groups

By the accepted statistical methods the standard deviation and probable error were calculated for each figure representing the mean number of intercalated discs in the given areas for each individual animal, and for each group of six animals at each age.[2]

The mean, probable error, and standard deviation of the number of discs occurring in twenty-five areas of 0.0085 sq.mm., for each animal in the eight normal groups, may be found in table 1. Table 2 contains for the eight normal groups and the two experimental groups, the mean, probable error, and standard deviation of the number of discs for the six animals of each age. A tabulation of the weekly per cent of increase in the number of intercalated discs from each age to 12 weeks is found in table 3. Figure 1 shows graphically the mean number of discs for each animal in the normal and experimental groups.

It is significant that the mean of discs present in the twenty-five areas for each animal at each given age differs only slightly from the mean of every other animal at that age. Furthermore, the mean of the number of discs for each animal at one age does not fall within the mean numbers of discs for any animal at any other age. There is but one exception to the above statement; the mean number of discs for animals nos. 122 and 133 at 2 weeks of age falls within the means for the animals at 1 week of age (table 1).

The probable error of the mean number of discs varies considerably for each animal. The variation, however, is not so great that it will cause an overlapping of any two groups of animals at different ages (table 2).

By examination of the figures in table 2, it may be seen that the greatest numerical increase in the number of discs per week occurs between the 3- and 4-week ages after birth. The increase here is approximately six discs, whereas it is

[2] Original counts for each individual animal are on file in the Smith College library.

TABLE 1

Mean, probable error, and standard deviation of the number of discs for each animal of the eight normal groups

ANIMAL NO.	SEX	AGE	MEAN AND PROBABLE ERROR	STANDARD DEVIATION
8		Late embryonic	1.12 ± 0.18	1.35
135	♂	Late embryonic	0.76 ± 0.15	1.10
136	♂	Late embryonic	0.80 ± 0.17	1.27
137	♀	Late embryonic	0.80 ± 0.14	1.06
138	♀	Late embryonic	0.64 ± 0.16	1.16
139	♂	Late embryonic	1.04 ± 0.21	1.59
7	♂	Newborn	3.52 ± 0.24	1.75
108	♀	Newborn	1.68 ± 0.14	1.05
131	♀	Newborn	2.32 ± 0.21	1.54
130	♂	Newborn	2.80 ± 0.31	2.30
132	♂	Newborn	1.68 ± 0.17	1.29
134	♂	Newborn	2.44 ± 0.18	1.33
9	♂	1 week	4.68 ± 0.31	2.31
101	♂	1 week	4.44 ± 0.22	1.60
107	♂	1 week	5.20 ± 0.37	2.73
109	♀	1 week	4.72 ± 0.28	2.07
128	♀	1 week	4.76 ± 0.41	3.05
129	♂	1 week	4.36 ± 0.35	2.59
10	♂	2 weeks	5.80 ± 0.17	1.30
100		2 weeks	6.12 ± 0.35	2.62
121	♂	2 weeks	5.48 ± 0.23	1.70
122	♂	2 weeks	4.48 ± 0.30	2.23
123	♀	2 weeks	6.56 ± 0.41	3.07
133	♂	2 weeks	5.16 ± 0.27	2.03
11	♀	3 weeks	8.68 ± 0.55	4.07
104	♀	3 weeks	7.32 ± 0.38	2.85
116	♂	3 weeks	7.32 ± 0.49	3.61
117	♀	3 weeks	8.68 ± 0.59	4.34
119	♀	3 weeks	7.44 ± 0.38	2.82
120	♀	3 weeks	8.20 ± 0.49	3.60
12	♂	4 weeks	15.40 ± 0.40	2.93
102	♀	4 weeks	14.24 ± 0.65	4.79
105	♂	4 weeks	14.04 ± 0.42	3.14
106	♀	4 weeks	14.08 ± 0.61	4.56
110	♀	4 weeks	12.76 ± 0.39	2.92
118	♀	4 weeks	12.96 ± 0.52	3.86
13	♂	8 weeks	20.80 ± 0.68	5.02
103	♂	8 weeks	21.88 ± 0.64	4.75
124	♂	8 weeks	20.76 ± 0.63	4.64
125	♂	8 weeks	19.92 ± 0.56	4.15
126	♂	8 weeks	21.12 ± 0.44	3.23
127	♂	8 weeks	21.04 ± 0.68	5.07
14	♀	12 weeks	28.16 ± 0.74	5.52
111	♀	12 weeks	27.16 ± 0.65	4.84
112	♀	12 weeks	27.68 ± 0.51	3.80
113	♀	12 weeks	26.20 ± 0.78	5.75
114	♂	12 weeks	27.36 ± 0.81	6.02
115	♂	12 weeks	25.52 ± 1.11	8.25

much less between any other two successive stages when calculated on a weekly basis.

Table 3 shows the weekly percentage of increase in the number of discs from each age to 12 weeks. The percentage of increase per week is greatest at the late embryonic stage, and becomes progressively smaller with the increase in age of the animal.

TABLE 2

The mean, probable error, and standard deviation of the number of discs in the eight normal and two experimental groups

GROUP 6 ANIMALS	AGE OF ANIMAL IN WEEKS	MEAN AND PROBABLE ERROR	STANDARD DEVIATION
1	Late embryonic	0.87 ± 0.04	0.15
2	Newborn	2.48 ± 0.15	0.55
3	1 week	4.70 ± 0.06	0.23
4	2 weeks	5.60 ± 0.15	0.56
5	3 weeks	7.96 ± 0.14	0.51
6	4 weeks	13.91 ± 0.20	0.73
7	8 weeks	20.97 ± 0.13	0.48
8	12 weeks	27.11 ± 0.22	0.79
9	Experimental 4 weeks thyroxin	14.06 ± 0.16	0.59
10	Experimental 4 weeks electric shocks	26.65 ± 0.83	3.02

TABLE 3

The per cent increase per week in the number of intercalated discs from each age to 12 weeks

AGES	PER CENT OF INCREASE
Late embryonic to 12 weeks	233.37
Newborn to 12 weeks	82.81
1 week to 12 weeks	43.36
2 weeks to 12 weeks	38.47
3 weeks to 12 weeks	26.75
4 weeks to 12 weeks	11.86
8 weeks to 12 weeks	7.32

The counts show, therefore, that there is a definite increase in the number of intercalated discs in the guinea pig from late embryonic stage to 12 weeks. The actual increase is greatest between 3- and 4-week ages after birth, and the percentage of increase is greatest at the late embryonic stage, thereafter becoming progressively smaller, when considered on a weekly basis.

Experimental groups

In table 4 may be seen the mean, probable error, and standard deviation of the number of discs for each animal of the two experimental groups. The animals, after experimentation of 2 weeks' duration, were 4 weeks old when killed, except animal no. X10 which was accidentally killed at 3 weeks.

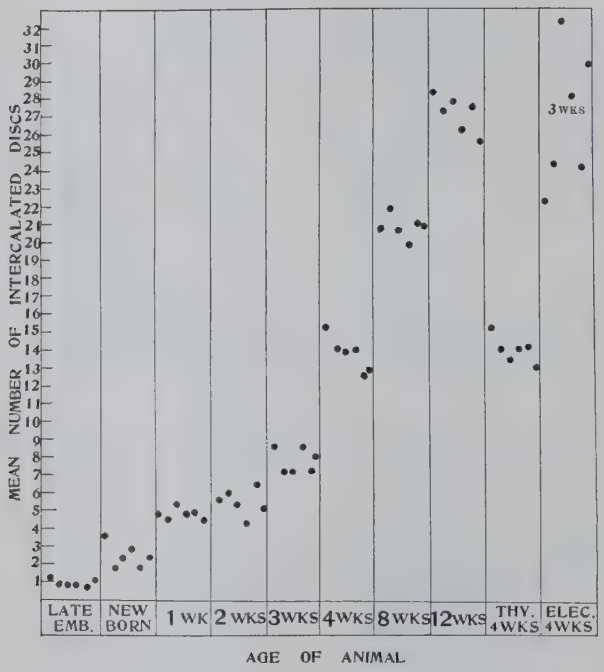

Fig. 1 Shows the mean number of intercalated discs for each normal and each experimental animal as given in tables 1 and 4.

The mean number of discs for each animal injected with thyroxin falls close to the mean number of discs for each animal in the normal 4-week-old group (table 1). The mean number of the normal 4-week-old group is 13.91 ± 0.20, whereas that of the thyroxin injected group is 14.06 ± 0.16. This indicates, therefore, that the injection of thyroxin had no appreciable effect upon the number of discs present.

The mean number of discs for each electrically stimulated animal is markedly higher than the mean number of discs for each control animal of the normal 4-week-old group (table 1). The means of animals nos. X1, X2, and X11 (table 4) are slightly less than those of the normal 12-week-old animals (table 1), and the means of animals nos. X9 and X12 (table 4) are slightly greater. The mean number of animal no. X10

TABLE 4

Mean, probable error, and standard deviation of the number of discs for each animal of the two experimental groups

ANIMAL NO.	SEX	AGE	MEAN AND PROBABLE ERROR	STANDARD DEVIATION
Thyroxin injected				
X3	♀	4 weeks	15.28 ± 0.68	5.04
X4	♂	4 weeks	14.24 ± 0.63	4.64
X5	♂	4 weeks	13.52 ± 0.56	4.12
X6	♂	4 weeks	14.08 ± 0.50	3.69
X7	♀	4 weeks	14.28 ± 0.92	6.80
X8	♀	4 weeks	13.12 ± 0.68	5.06
Electrically stimulated				
X1	♀	4 weeks	22.24 ± 0.48	3.59
X2	♀	4 weeks	24.28 ± 0.87	6.48
X9	♀	4 weeks	32.40 ± 1.20	8.92
X10	♀	3 weeks	27.88 ± 0.88	6.55
X11	♀	4 weeks	24.00 ± 1.05	7.75
X12	♀	4 weeks	29.60 ± 0.70	5.20

is 27.88 ± 0.88, or approximately four times that of the normal 3-week-old animal, which is 7.96 ± 0.14. The mean of the normal 4-week-old group is 13.91 ± 0.20, whereas that of the electrically stimulated group is 26.65 ± 0.83 (table 2).

DISCUSSION

The generalization of earlier investigators (Aschoff, '06; Cohn, '09; Jordan, '11; Jordan and Banks, '17; and Witte, '19) that there is a definite increase in the number of intercalated discs in heart muscle with an increase in the age of the animal is confirmed by counts (table 1). A single method, Zimmermann's technique, was employed on all the material

used. Any variation in the number of discs due to differences in fixatives and stains, mentioned by Jordan ('11) and Körner ('33), is therefore ruled out; and the per cent increase in the number of discs with age is significant. The number of discs appearing in guinea pigs of the same age is remarkably constant. The findings substantiate Marceau's theory ('04) that the number of intercalated discs is almost constant for a given species of animal at the same age and in the same region of the heart. Furthermore, the sex of the animal does not affect the number of discs present in heart muscle of the guinea pig. Kato ('28) also found this to be true in the rabbit.

Buhlig ('02) and Okamura ('30) maintained that intercalated discs are artefacts. This interpretation is not tenable since the number of discs is significantly constant in animals of the same age and shows a progressive increase with the age of the animal. Moreover, in the group of electrically stimulated animals the number of discs not only increases, but this increase is found to be chiefly in a certain type of disc, namely, the band form.

Thyroxin, injected into six animals, produced no increase in the mean number of discs as compared with those present in normal animals of the same age (table 2). In this experimental work, we followed the method of Yater ('31) who reported an increase in the rate of heart beat of 38 per cent in the rabbit. Without further experimentation in varying the amounts of thyroxin administered, no deduction can be made regarding its effect upon the rate of heart beat in the guinea pig or, in turn, the effect of the rate of heart beat upon the number of discs present.

Electrical stimulation of six 2-week-old animals over a period of 2 weeks caused a very marked increase in the number of discs present as compared with those in normal animals of the same age (table 2). Jordan and Steele ('12) reported an unsuccessful attempt to change the number of intercalated discs in heart muscle by electric stimulation but did not describe their procedure. What factors are involved

in producing an increase in the number of discs is not entirely clear. Mangin's observations ('31) point to a correspondence of the number of discs present in a given region of the heart with the force of contraction of that region. The electrical stimulation caused a decided increase in the activity of the animal and thus increased the force as well as the total amount of the work done by the heart. The increase in the number of discs present in the electrically stimulated animals would seem to support Jordan's explanation ('33) that the discs are irreversible contraction bands. Since increased muscular exercise is accompanied by an increase in the formation of lactic acid, an irreversible coagulation may be brought about, as Jordan has suggested, thus preventing the normal process of relaxation from taking place, and causing 'irreversible contraction bands' to appear as intercalated discs.

SUMMARY

1. Actual counts of the intercalated discs occurring in a specified area of ventricular muscle of the guinea pig reveal the following points.

a. The number present at a given age is remarkably constant.

b. The sex of the animal does not affect the number of discs present.

c. The number increases with the age of the animal.

d. The percentage of increase in number is greatest at the late embryonic stage, becoming progressively less with the increase in age of the animal.

e. The greatest numerical increase occurs between the 3- and 4-week ages after birth.

2. Injections of thyroxin, as administered in the experiments reported, produce no appreciable effect on the number of intercalated discs present.

3. Electrical stimulation of the living animal increases the number of discs present to approximately twice that of the normal guinea pig of the same age. The increase is chiefly in the number of band forms of discs present.

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THE TRANSPARENT CHAMBER ADAPTED FOR CELL CULTURE AND PERMITTING ACCESS TO THE CONTAINED LIVING TISSUE [1]

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ONE FIGURE

The original procedure for introducing cells into a transparent chamber inserted in a rabbit's ear was described by Clark et al. in 1930. This consisted in using a 'round table type' chamber provided with an oblique hole in the table opening on the growing region which, when not in use, was occluded by a glass plug sealed in with paraffin. Small pieces of tissue could be introduced from below through this hole and deposited either above, in, or below the thin layer of growing tissue. This was technically a difficult method and in many ways unsatisfactory. To simplify the handling and introduction of minute transplants Dr. H. T. Kirby-Smith developed a form of micromanipulator. With such an instrument very small bits of tissue could be handled. However, the proportion of unsuccessful implantations was very great and it was thought that the technique should be simplified. Accordingly, a chamber was constructed in such a manner that when inserted in the ear the central growing region could be exposed from above without disturbing the assembly in the ear. The tissue could then be covered again and the chamber allowed to continue its normal course. This process could be repeated many times on the same specimen.

[1] The work in this laboratory on the development of methods for studying living cells and tissues in transparent chambers installed in the living mammal is being aided by a grant from the Rockefeller Foundation.

The type of chamber here described is the 'pre-formed' tissue type (Clark et al., '30). It is used for studies where it is desirable to deal with the original tissues of the ear. The 'round table type' may be used when it is essential to deal with newly formed tissues.

The chamber is constructed of celluloid. This material may be obtained from the Eastman Kodak Company in thicknesses up to 0.5 mm. under the trade name Kodaloid. The thicker pieces are sold as 'Viscoloid' by the DuPont Viscoloid Company. The various parts of the chamber are shown in figure 1. To make the top, a ring of viscoloid is prepared having an outside diameter of 3.5 cm. and an inside diameter of 2 cm. The inside edge is beveled on a grinding wheel. Over the hole in this ring a piece of clear mica 0.004 inch thick and 2.4 cm. in diameter is glued. The glue used is that developed by Varian ('31). Three holes are drilled in the viscoloid ring as shown in figure 1.

The bottom or observation side of the chamber consists of two parts. Part 1 is a ring cut from viscoloid 1.2 mm. thick having the dimensions given in figure 1. To the inner margin of this piece another ring of viscoloid 2 mm. high and 2 mm. thick is fused with a mixture of equal parts of absolute alcohol and ether. On top of this ring a circular piece of celluloid 0.5 mm. thick, with an outside diameter of 3.3 cm. and an inside diameter of 1.6 cm., is fused in a similar way. The thickness of the piece is then reduced to 0.36 mm. by rubbing it against a piece of 00 sand paper. Three holes are bored in the outer ring to correspond to the three holes in the top. Three equally spaced holes are then drilled through the thick part of this piece (fig. 1), using a no. 55 twist drill in a high speed drill press. The inner ends of these holes are countersunk to receive the heads of three brass bolts which have been ground square and reduced to less than 0.5 mm. in thickness. The bolts are forced in place and the alcohol and ether mixture applied around them. Pieces of thin celluloid, 0.075 mm., are then fused over the heads so that they will not come in direct contact with the tissue when the chamber is installed.

Part 2 of the base is formed of celluloid rings having an outside diameter of 2.3 cm. and an inside diameter of 1.7 cm. stacked one on another until a height of 3 mm. is reached. On top of this a ring of celluloid, 1.2 mm. thick, with an outside diameter of 3 cm. and an inside diameter of 1.7 cm. is placed

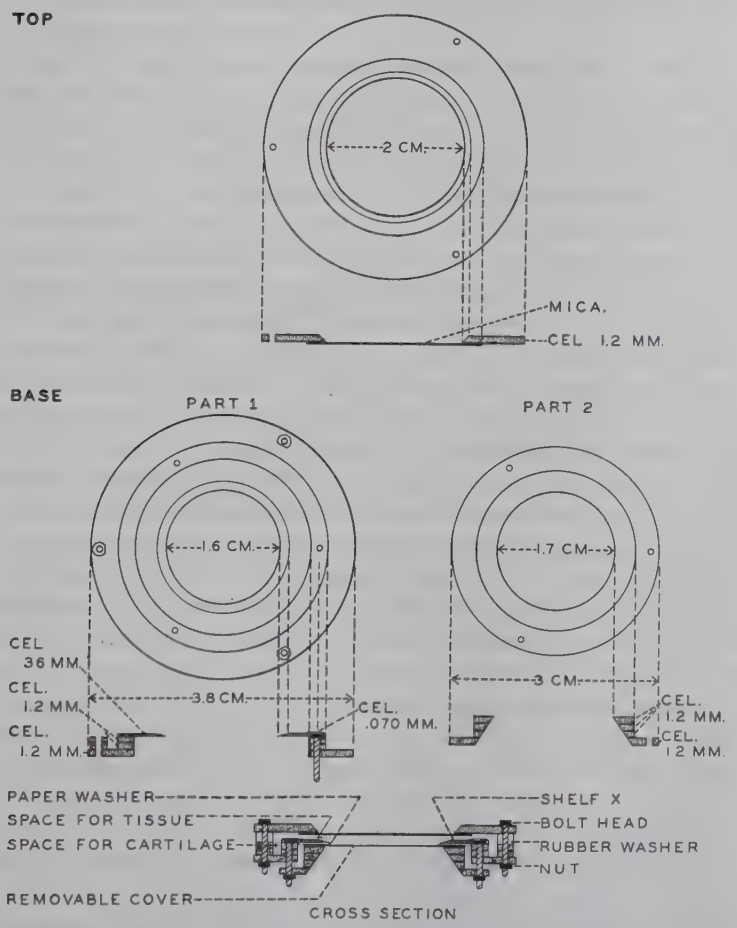

Fig. 1 Diagram giving the materials and dimensions for constructing the chamber. *Cel.* refers to celluloid. Figures given in millimeters represent the thickness of the various pieces. Natural size.

and all pieces are fused together with alcohol and ether. The inside edge of this piece is then beveled with a small grinding wheel or a bastard file, as shown in figure 1. The overlapping portion of this part is drilled with a no. 54 twist drill at three points corresponding to the protruding bolts in part 1.

A paper washer is cut from bond paper so that it fits snugly on the shelf x (fig. 1). The cover is made from clear mica about 80 μ thick and rests on the paper washer on the shelf x. These parts are held in place by the removable collar, part 2 (fig. 1), which is in turn held down by the nuts and bolts installed in part 1 (fig. 1).

Preliminary to the installation in the ear the various parts of the chamber are immersed in 1:500 metaphen for 20 minutes, except for the paper washer, which is immersed in a smoking hot, but not boiling mixture of bayberry wax and vaseline, 1 to 3. Just before starting the installation the parts of the chamber are removed from the metaphen, washed in distilled water and dried. The paper washer is recovered from the wax mixture and, when the wax has solidified, is placed on the shelf of the base element. The mica cover is placed on top of it and pressed down. The retaining collar is then applied and the nuts screwed down. A water- and bacteria-tight joint is thus made between the removable cover and the rest of the chamber. For the formula of the wax mixture I am indebted to Dr. S. C. Williams, of this laboratory. From this point on the installation of the chamber is the same as that described for other 'pre-formed' chambers (Clark et al., '30).

Such a chamber when installed in an ear follows the same course as is usual for the 'pre-formed tissue' chamber. After the immediate effects of the operation have disappeared, the cover may be removed and the chamber contents exposed. The following precautions must be observed: The entire ear and chamber are sterilized by immersion in metaphen, 1:500, or some similar antiseptic which neither injures the delicate tissues of the ear nor affects the celluloid. All subsequent operations are carried out aseptically. The nuts over the retaining collar are removed. The well in the chamber is then

filled with cool Ringer's solution, about 60°F. If the entire procedure can be done in a 50°F. constant temperature room, so much the better. The retaining collar is now removed. The mica cover stays on the paper washer, and can be raised with a needle. This should be done very slowly, allowing the Ringer's solution to fill the space formed by raising the cover, which can shortly be removed with forceps. The thin tissue is then exposed under fluid and if the operations are done slowly enough and at the proper temperature, no hemorrhage occurs. Under optimum circumstances the tissue can be exposed for as much as 1 hour and no deleterious effects occur. However, should the temperature be too high, swelling occurs and much damage will be done the preparation. This swelling can be decreased by a preliminary injection of ephedrin into the base of the ear. The replacement of the cover is the reverse of taking it off. If there has been any swelling the collar must be screwed down slowly, taking as long as 1 to 2 hours to reach the original position of the nuts.

For the application of the external protective splints and shields which have added much to the success of the transparent chamber technique and for the routine handling and care of the chambers, the reader is referred to the article by Clark and Clark ('32, p. 55).

This project, which extends the possibilities for studying living cells in vivo, was done under the stimulus of Dr. E. R. Clark, who developed the transparent chamber methed in connection with his researches on living cells in the living animal.

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AN ADAPTATION OF THE TRANSPARENT CHAMBER TECHNIQUE TO THE SKIN OF THE BODY[1]

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THREE FIGURES

A device for studying in situ living mammalian tissues which could not otherwise be studied with high magnifications was first worked out by Sandison, under the direction of Dr. E. R. Clark, and described by him in 1924. His apparatus consisted of a double, thin-walled, transparent chamber with a space between the walls into which tissue grew when it was inserted into the ear of a rabbit. This original procedure has been perfected and extended by a group of workers in this laboratory, Clark, Kirby-Smith, Rex and Williams ('30).

Thus far the installation of chambers has been confined almost exclusively to the ears of rabbits. In connection with certain studies on nerves, it seemed desirable to attempt the installation of chambers in another region of the animal—one having a nerve supply more easily accessible for operation than the ear. Such a place would also provide a means of comparing the growth and behavior of the cells and tissues of the ear with those of another part of the animal. At the suggestion of Dr. E. R. Clark, the following method was evolved.

The technique consists in making a skin flap on a rabbit in such a manner that it receives its blood and nerve supply from one end and is free at the other. This flap is sufficiently

[1] The work in this laboratory on the development of the method for studying cells and tissues by the employment of transparent chambers installed in the living animal is being aided by a grant from the Rockefeller Foundation.

long to allow for the installation of a chamber in the free end and to permit study of the chamber under the microscope.

A rabbit is prepared for operation by shaving the hair from one side of the back from shoulder to the tail and washing the region with green soap solution. Fifty mg. of sodium amytal per kilogram of body weight is then injected intraperitoneally. When anesthesia is complete a celluloid strip 11 inches long and $2\frac{1}{2}$ inches wide is sewed to the skin about 1 inch from the vertebral column. The silk sutures are 1 cm. apart and extend entirely around the strip. The purpose of

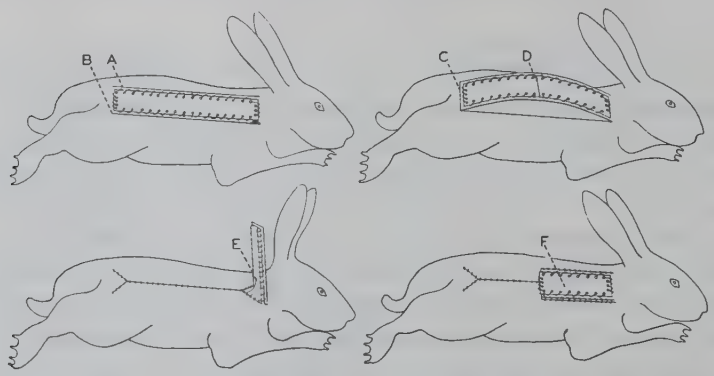

Fig. 1 Diagrams illustrating four stages in the formation of a flap. A, B and C refer to skin incisions; D marks the point at which the celluloid pattern is cut, E indicates the manner in which skin edges are sutured and F represents the position of the flap against the body.

this strip is to prevent retraction of the skin following cutting and thereby facilitate the handling of the skin during the formation of the flap, and also to act as a protective splint. Incisions are made through the skin on the sides of the strip just outside the suture lines (fig. 1, A and B). The panniculus carnosus muscle and the segmental nerve supply to this region are exposed through these incisions. The nerves are the lateral branches of the posterior divisions of the intercostal nerves and the posterior divisions of the lateral branches of the same nerves together with branches from the lower cervical nerves. As many of these nerves as possible, generally

the eighth cervical and the first, second and third thoracic nerves, are dissected free from the underlying structures so that they may be incorperated in the base and insure a nerve supply to a portion of the flap. The incisions are then carried through the remaining structures down to the body muscles. Inspection is made of the undersurface of the incised skin to see that sufficiently large blood vessels enter it at the shoulder end. The caudal end is then cut across (C, fig. 1). The celluloid strip is cut as at D (fig. 1), and the distal part bent back on the proximal part as at E (fig. 1). The skin edges are approximated with small metal clips and the extensive defect in the body skin closed by drawing the skin edges together (fig. 1). The tail end of the defect must be closed with silk sutures so that the suture line will be strong enough to withstand the added tension on the skin when the animal sits upright. The inside splint may then be removed and the flap laid back against the body. It is held in place by a gauze bandage 6 inches wide extending around the thorax. Over this a muslin bandage is sewed.

The flap is inspected at weekly intervals. At the end of the first week the metal clips are removed. The silk sutures are not removed. The edges are cleaned with metaphen, 1:500, and the bandage reapplied.

After 2 weeks the wound is sufficiently healed and a chamber may be inserted. The type of chamber used is that which has been called the 'round table' chamber, Clark et al. ('30), differing from the described form in that the table is built up to 3 mm. in height.

An area of skin the size of the top element of the chamber is dissected off the outside of the flap about $\frac{3}{4}$ inch from the free end. A corresponding area is removed from the opposite surface. All dissections are done with a sharp knife. In the center of this denuded region a stab wound is made through and through, and puncture holes made for the washers around the bolt holes. The bottom element of the chamber is then inserted from the inside. The top element is applied and the two parts held together in the flap by three bolts and nuts.

The tension on the mica cover is adjusted so that the 'buffers' on the table are firmly in contact with it, but are not causing it to bulge.

In the ear the cartilage serves to anchor the chamber in place, but in addition external protective splints are used to absorb any shocks which would otherwise act on the chamber. The flap chambers are even more susceptible to outside violence, since they are inserted in a flabbly tissue which has no supporting cartilage. Hence some form of splinting is essential. The method of doing this consists in applying kodaloid strips 0.028 inches thick to each side of the flap, holding them in place temporarily by a bolt at each corner where the strip extends beyond the flap. After a few days, the kodaloid is firmly adherent to the skin and the bolts may then be abandoned. Where the kodaloid comes in contact with the top of the chamber, it is cut away to permit subsequent microscopic examinations.

The flap is held in place against the body as previosly described. All operative procedures are carried out with strict asepsis.

The chamber is allowed to remain undisturbed for 8 days. By this time the growth has invaded the thin space in the center of the chamber and the preparations may be studied daily for as long as necessary. Figure 2 is a photomicrograph of the flap in position for study on the stage of the microscope.

The animal board used for holding the animal when the flap is studied must be narrow. It consists of a board $4\frac{1}{2}$ inches wide to the sides of which two pieces of thin wood are nailed so that a trough is formed in which the animal lies on its back. There is a side opening through which the flap projects. A head stock fits over the neck and nails at the foot provide places to which the hind legs may be tied. The board is raised from the table by end pieces as high as the stage of the microscope.

When the daily observations are begun, a simpler method for retaining the flap against the body is used. This consists of a kodaloid box with a removable top into which the flap is

placed loosely and the whole held against the animal by a piece of muslin and safety pins.

Ten such flaps have been made and chambers installed in eight. In every case growth has appeared on the table and in one new tissue has grown entirely across. Figure 3 is a photograph showing the amount of growth 17 days after installation of the chamber. The fate of the growth, once it has appeared on the table, is largely determined by the degree of immobilization of the chamber. When properly immobilized by external splinting and when handled carefully

Fig. 2 A photograph of a flap with the installed chamber under a microscope.

the time of appearance of the growing vessels and their rate of growth parallels that for the ear very closely. No detailed comparison between the two can yet be made, as sufficient data concerning the flap have not been obtained. Vessels, both blood and lymph, invade the chamber in the same manner here as in the ear. No nerves have yet been seen in these flap chambers. However, if the flap is prepared properly, the area in which the chamber is placed responds to sensory stimuli. Such being the case, it would seem from the experience with ear chambers in this laboratory that nerves will grow in.

The method described above extends the usefulness of the transparent chamber technique by applying it to the body skin. The nerve supply to the skin of the body is easily accessible for operation, which is not the case with the nerve supply to the ear. Hence certain types of experimentation amenable

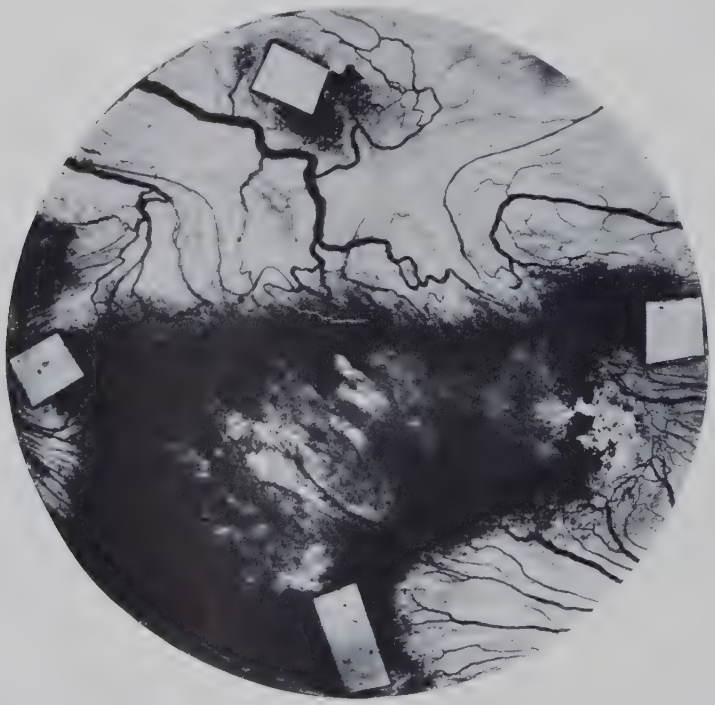

Fig. 3 A photograph taken 17 nays after the installation of the chamber. The four small quadrilateral areas are the 'buffers' which maintain the thickness of the observation space. The dark area is the part of the chamber as yet uninvaded by the new tissue. × 16.

to the transparent chamber technique may be done more readily in flaps. It is possible when installing the chamber in a flap to place a portion of panniculus carnosus muscle on the table and provide an opportunity for studying living striated muscle. Up to the present time, the transparent chamber technique has been applied only to animals having sufficiently

long ears. By the use of flaps the number of kinds of animals which may be used for study can be considerably increased.

A type of chamber equipped with a removable top has been devised which is described previously.[2] When this type of chamber is used in a flap, a favorable place for implanting other tissues is provided, since the temperature of the flap can be maintained at body temperature.

[2] See page 487 in this number of The Anatomical Record.

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ADDITIONAL EVIDENCE OF THE FAILURE OF EPIPHYSEAL UNION IN THE SKELETON OF THE RAT. STUDIES ON WILD AND CAPTIVE GRAY NORWAY RATS

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TWO PLATES (TWELVE FIGURES)

In an earlier paper (Dawson, '34) it was pointed out that observations on the state of diaphyso-epiphyseal union in several varieties of mature rats should be made to determine whether the condition of lapsed epiphyseal union is restricted to certain domesticated strains (Wistar, Bussey) or is characteristic of the rat (Dawson, '25, '27, '29). An opportunity to extend such observations came sooner than was anticipated. Through the kind offices of Prof. H. H. Donaldson, bones of the captive gray (King and Donaldson, '29) and wild gray Norway rats have been placed at my disposal. I wish to express my indebtedness to those who have made this material available for study.

Only the humerus and femur were included in the material loaned, but this did not constitute a serious handicap. Earlier studies had shown that the upper end of the humerus and the lower end of the femur were relatively constant in their behavior so far as epiphyseal union, or rather failure of epiphyseal union, was concerned. The head of the femur is more variable in this respect, and at the lower end of the humerus the epiphysis is united early in the second month of life. Accordingly, in this study interest centers chiefly about the head of the humerus and the lower end of the femur, since they may be regarded as furnishing an index of the general

situation in the skeleton as regards lapsed epiphyseal union. The head of the femur, although not so valuable an indicator, also furnishes supplementary evidence.

Bones from twelve animals (six males and six females) of the captive gray strain were studied. The range in age was from 554 to 627 days. Twenty-four wild gray rats (thirteen

EXPLANATION OF TABLES 1 AND 2

These tables give the available data on the animals from which bones were taken. Ph. = prepared in aqueous solution of phenol. G. D. = prepared with commercial Gold Dust. Where bones have been sawed longitudinally to check the observations made on their surfaces, this fact is indicated under remarks.

TABLE 1

King's captive gray strain of rats

	SEX	AGE IN DAYS	BODY WEIGHT	BODY LENGTH	METHOD OF PREPARATION	REMARKS
			grams	*mm.*		
1	♀	554	360.6	232	G. D.	Humerus head sectioned; open
2	♀	599	308.5	232	Ph.	
3	♀	609	391.0	242	Ph.	Lower end femur sectioned; open
4	♀	612	315.0	221	G. D.	
5	♀	614	271.0	233	G. D.	
6	♀	616	338.0	233	Ph.	
7	♂	595	318.0	230	Ph.	
8	♂	599	358.6	241	Ph.	
9	♂	612	476.0	246	G. D.	Humerus head sectioned; open
10	♂	615	414.0	256	Ph.	Femur head sectioned, completely fused; lower end and humerus head open
11	♂	616	460.5	254	G. D.	Lower end femur sectioned; open
12	♂	627	354.0	237	G. D.	

males and eleven females) were also represented in the collection of bones. The ages of these animals are unknown and usually only body weight was recorded. Detailed information regarding both the captive and wild grays is recorded in tables 1 and 2.

The bones were cleaned in two ways: some in hot 2 per cent commercial Gold Dust, others in an aqueous solution of phenol. Both methods of preparation appear adequate. After

Gold Dust the entire bone is white, while after phenol the bone is pink or brown with the line of disjunction between epiphysis and shaft deeply stained. The entire bones were first studied carefully with brilliant illumination under a high-power binocular dissecting microscope. Representative bones

TABLE 2

Wild gray rats

	SEX	BODY WEIGHT	METHOD OF PREPARATION	REMARKS
		grams		
1	♀	250.8	G. D.	
2	♀	274.0	G. D.	
3	♀	282.0	G. D.	
4	♀	282.7	G. D.	
5	♀	304.7	G. D.	
6	♀	313.7	Ph.	
7	♀	321.8	Ph.	
8	♀	346.6	G. D.	
9	♀	390.0	G. D.	Epiphysis of femur head shelled off in sawing
10	♀	480.3	G. D.	
11	♀	482.5	G. D.	
12	♂	221.7	G. D.	
13	♂	259.6	Ph.	
14	♂	303.0	G. D.	
15	♂	306.6	G. D.	
16	♂	339.3	G. D.	
17	♂	347.9	Ph.	
18	♂	354.3	Ph.	Femur head sectioned; open
19	♂	354.7	Ph.	
20	♂	357.4	G. D.	Epiphysis of femur head shelled off in sawing
21	♂	436.1	G. D.	Humerus head, lower femur sectioned; open
22	♂	438.6	G. D.	Both ends of femur sectioned; open
23	♂	461.3	G. D.	
24	♂	640±	G. D.	Femur head sectioned; open

were then photographed. Later longitudinal sections were made of some of the bones with a fine saw. Some of these were ground smooth on a carborundum stone, others left rough. Grinding was discontinued as it was found to disrupt the line of disjunction and obscure detail. When ground wet and removed from the holding matrix the epiphysis sometimes separated from the shaft, due to the swelling of the cartilage following absorption of water.

Bones prepared with phenol were relatively soft and were sawed without any chipping, while those prepared with Gold Dust were hard and brittle. In the head of the femur after the saw had passed through the epiphysis, one-half of it occasionally shelled off from the shaft. In other instances peripheral fragments of the epiphysis were broken away from the shaft by the action of the saw. In relatively fresh bones cleaned in the phenol solution, fat was still present in the marrow, and at the epiphyseal line the arrangement of the cartilage cells could be distinguished under adequate illumination and magnification.

Discontinuity between the shaft and epiphysis is evident in all cases in the upper end of the humerus (figs. 1, 4, 7, 10) and the lower end of the femur (figs. 3, 6, 9, 11, 12). In some instances, however, the line of separation cannot be followed completely around the bone. At the head of the humerus this line is partially obliterated by what appears to be an overgrowth from the deltoid tuberosity while in the femur the line is frequently obscured on the popliteal surface. In both instances these conditions appear to be the result of secondary processes unrelated to the activity at the zone of disjunction. When viewed in section, the bone covering the line of separation appears to be a superficial addition, probably of subperiosteal origin. The epiphyseal line does not appear to be modified in such cases except peripherally in the zone of overgrowth.

The line of separation between the epiphysis and shaft at the head of the femur is not always so readily distinguished. In some instances this is due to partial or complete fusion of the epiphysis. In many other cases, however, the margin of the epiphysis has overgrown the diaphysial portion of the head and its free edge has rolled in toward the neck. The line of separation is then no longer visible on the surface. In figure 2 the line of separation is distinct, in figure 3 it is not so clearly seen, and in figure 8 it is practically completely obscured. However, sawed sections of the last show a well-defined epiphyseal line. Yet in a femur of almost the same

appearance, from a captive gray rat 615 days old, sectioning revealed a completely fused epiphysis with all traces of the epiphyseal line lost and continuous trabeculae passing from shaft to epiphysis. The variability in the behavior of the epiphysis of the femur head was noted earlier in the Wistar rats (Dawson, '29, p. 115).

DISCUSSION

These observations on captive and wild gray rats, although covering only the humerus and femur, are sufficient to indicate that lapsed epiphyseal union at certain points of the skeleton is as characteristic for the gray rat as for the Wistar albino and Bussey hooded strain. For the captive gray rats adequate data as to age, sex, body weight and body length are available. For the trapped, wild grays only sex and body weight are known. It appears, furthermore, almost impossible to make any reliable estimate of their ages from the data on the captive rats (King, '23); there is no close correlation between age and body weight (table 1). It seems probable that some of the wild animals had attained ages comparable to those of the captive strain, for instance, the male weighing 640 gm. (no. 24, table 2).

It seems unnecessary to discuss again in detail the implication of these findings (Dawson, '29). Todd ('33), however, still remains skeptical as to the widespread occurrence of lapsed epiphyseal union in rats. His examination of the bodies of 150 wild rats (Muridae) indicated clearly that the epiphyses close and unite in a definite order as they do in other mammals. It is difficult to explain this discrepancy between our observations and it must be left until further studies have been made. The present observations, however, lead me to reiterate the statement already made (Dawson, '34) that hereditary rather than environmental factors are responsible for the condition of epiphyseal union in rats. Naturally, environment cannot be ruled out entirely, but the fact that lapsed epiphyseal union occurs in four different strains of rats, two under long domestication, one under rela-

tively short domestication, and a wild strain, strongly suggests that the deficiency leading to abnormal differentiation of the skeleton is inherent in these animals. Environment may possibly be operative in producing a marked expression of this deficiency, but if it were as potent as has been suggested (Todd, '33), lapsed union should be encountered more frequently in many other animals (Todd, '30, '31). It is unlikely that an unfavorable environment would bear more heavily upon the rat than on other mammals.

SUMMARY

The status of epiphyseal union has been determined in the humerus and femur of twelve King's captive gray, and twenty-four wild gray Norway rats. Cleaned and dried material was used. The animals from King's colony ranged in age from 554 to 627 days. Only the body weight of the wild grays was known. In all instances the epiphyses of the proximal ends of the humerus and the distal end of the femur were not fused. At the head of the femur more variation was encountered, but in many instances the epiphyses were still ununited.

These observations taken in conjunction with those already reported for the Wistar albino and the Bussey pink-eyed hooded rats indicate that failure of epiphyseal union in certain bones is due to hereditary rather than environmental factors.

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EXPLANATION OF PLATES

All figures are photographs of dry bones, magnified approximately six times. Figures 1 to 9 are surface views. Different surfaces were photographed so that a more general impression of the appearance of the bones could be given. Figures 10 to 12 are sawed, unpolished sections.

PLATE 1

EXPLANATION OF FIGURES

1 to 3 Head of the humerus, head of the femur and distal end of the femur, respectively, of a captive gray male Norway rat from King's colony; body weight, 354.0 gm.; body length, 237 mm.; age, 627 days. Evidences of diaphyso-epiphyseal discontinuity are distinctly seen.

4 to 6 Head of the humerus, head of the femur, and distal end of the femur, respectively, of a wild gray female Norway rat; body weight, 482.5 gm. Diaphyso-epiphyseal disjunction is clearly seen in the head of the humerus and lower end of the femur. In the head of the femur the line of separation cannot be followed completely around the bone.

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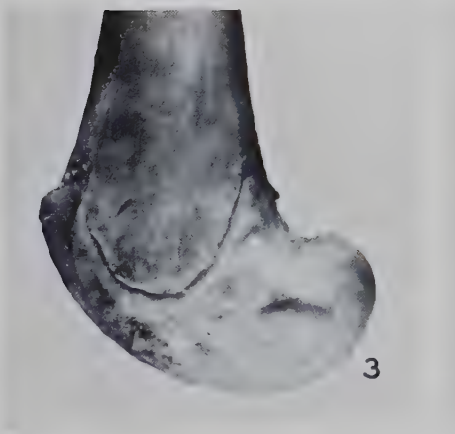
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PLATE 2

EXPLANATION OF FIGURES

7 to 9 Head of the humerus, head of the femur and distal end of the femur, respectively, of a wild gray male Normay rat; body weight, 640 gm.; body length, 269 mm. Diaphyso-epiphyseal separation is distinct in the head of the humerus and lower end of the femur, but is not evident in the head of the femur. Sawed sections, however, show a distinct epiphyseal line in the femur head.

10 and 11 Sawed sections of the head of the humerus and distal end of the femur of a wild gray male rat; body weight, 436.1 gm. The persistent line of junction between the diaphysis and epiphysis in both bones is shown. In the opposite half of this end of the femur the epiphysis was separated from the diaphysis while smoothing the surface of the section.

12 Sawed section of the lower end of the femur of a captive gray male rat from King's colony; body weight, 460.5 gm.; body length, 254 mm.; age, 616 days. The line of junction is distinctly seen.

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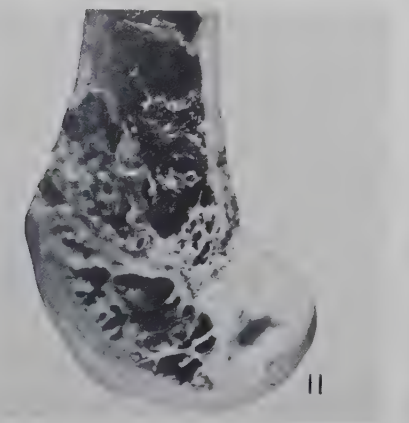
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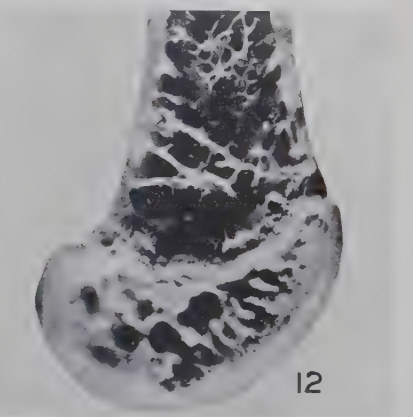
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